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Antiangiogenic and morphological effects of *Cinnamomum cebuense* Kosterm. leaf extracts on *Anas platyrynchos* L. embryonic development usingan *in vivo* chorioallantoic membrane (CAM) assay

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Abstract

The theoretical efficacy of antiangiogenic treatment in tumor growth and other angiogenesis-dependent diseases offers a promising approach that leads to the robust search of angiogenesis inhibitors. *Cinnamon* species, widely used in food processing and other commercial industries, are among the medicinal plants currently being exploited by many cancer researches that involve inhibition of angiogenesis. The Cebu endemic *Cinnamomum cebuense* is popular for its bark as a remedy for stomachache, headache and toothaches. Phytochemicals found in many cinnamon species worldwide that were claimed to inhibit angiogenesis and tumor cell proliferation were also found in *C. cebuense*. However, there have been no reported studies that verified its antiangiogenic activity. In the present study, the *C. cebuense*' santiangiogenic property was investigated via CAM assay with five different treatments of *C. cebuense*'s leaf aqueous extract (CCE) following a completely randomized design: 100% (T1), 50% (T2), 25% (T3), 12.5% (T4), and 6.25% (T5). Sixteen-day old duck eggs were harvested after 15 days incubation, embryos assessed morphologically, angiogenesis quantified by photo documentation of the CAMs, and fractal dimension index analyzed using Image J analysis software. Results revealed that crown to rump length (CRL), hindlimb, forelimb, and beak lengths for other treatments were not dose-dependent except for T1 (100%) where embryos were clearly stunted in all its morphological development. The lowest mean fractal index (MFI=0.3042) among the experimental groups was also evident in T1 (100%). The results show strong evidence that T1 (100%) is the most potent concentration for CCE that could inhibit angiogenesis, and thus possibly restrain proliferation and metastasis of cancer cells.

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Introduction

Herbal plants have been utilized tremendously since time immemorial and served as the cornerstone for the development of new drugs (Vickers *et al*., 2001). The demand for plant-based medicine remains strong as they are generally regarded as cost effective, safer, and less harmful than synthetic drugs (Benzie and Galor, 2011). Cinnamon, a highly prized spice plant, is among the herbal plants utilized by the locals in Cebu Island, Philippines. Aside from being a good source of essential oils and timber (Ravindran *et al*., 2004), it is also used as a remedy for gastric pains (Del Fierro *et al*., 2012), an antispasmodic (Jaafarpour *et al*., 2015), aromatic, astringent, antiseptic, germicidal, and a remedy for flatulence (Vijayan and Thampuran, 2004). There are 21 species of cinnamon that can be found in the Philippines, 16 of which are known to be endemic (Ragasa *et al*., 2014). *C. cebuense* Kosterm.is one of those endemic cinnamon trees that can only be found in Cebu Island, Philippines and it is commonly called as *kaningag* or *kalingang*. Its bark is popularly stripped for stomach ache, headache, and toothache remedies (del Fierro *et al*., 2012).

Angiogenesis is a multistep, advanced, physiological process in which new vasculature is formed from pre-existing vasculature (Deryugina and Quigley, 2008). Angiogenesis, especially if it is regulated, is vital to normal physiological processes like wound healing and embryonic development (Sagar *et al*., 2006; Penn, 2008; Irvin *et al*., 2014). Either excessive or insufficient blood vessel growth leads to a growing list of diseases: cancers, psoriasis, arthritis, diabetes, obesity, asthma, cardiovascular disease, stroke, and many others (Tahergorabi and Khazaei, 2012). The concept that tumor growth is angiogenesis-dependent and that inhibition of angiogenesis could be therapeutic was proposed by Judah Folkman in 1971 (Ribatti, 2008).Since then, there was an unprecedented search for compounds that inhibit angiogenesis as well as continuous evaluation in clinical trials.

Cinnamon possesses antiangiogenic potentials and hence being exploited in many cancer researches that involve angiogenesis inhibition by blocking certain vital steps (Vijayan and Thampuran, 2004). In a study conducted by Thanekar *et al.* (2016), *C. tamala* blocked tumor growth by inhibiting angiogenesis. *C. zeylanicum* obstructing vascular endothelial growth factor receptor - 2 (VEGFR2) kinase and stopping vascular endothelial growth factor (VEGF)-needing activities inhibited angiogenesis, thus blocking tumor growth (Zhang *et al*., 2009). *C. cassia* also exhibited antiangiogenesis in zebrafish bioassay and human umbilical vein endothelial cell (HUVEC) assay by inhibiting VEGFR1 and VEGFR2 (Bansode *et al*., 2012). Other studies reported that cinnamon extract can initiate apoptosis on active cancer cells (Kwon *et al*., 2010); and 2'- hydroxycinnamaldehyde from the bark of the cinnamon strongly inhibited the growth of gastric and colon cancer cell lines (Jin Won *et al*., 1994).

Phytochemical screening of *C. cebuense*'s leaves and barks revealed the following chemical constituents: eugenol, squalene, monoterpene, sesquiterpene, α-terpineol, β-caryophyllene, triterpenes, safrole, trilinolein, humulene, polyprenol, 4-allyl-2-methoxyphenol, 4-hydroxy-3-methoxycinnamaldehyde, α-amyrin, β-amyrin, and bauerenol (Del Fierro, 2012; Ragasa *et al*., 2013; Espineli, 2013). β-amyrin and α-amyrin are pentacyclic triterpenes that exhibit anti-inflammatory effect (Basile *et al*., 1988) and reduce VEGF expression by immunohistochemistry (Bento *et al*., 2009). Humulene is a terpene that exhibited an anti-cancer activity and is enhanced with the combination of β-caryophyllene (Legault and Pichette, 2010).

Eugenol helped stabilize membrane on synaptosomes, erythrocytes, and mast cells (Prakash and Gupta, 2005), while Manikandan *et al.* (2010) reported that eugenol initiated apoptosis which lead to inhibition of angiogenesis. Cinnamaldehyde, a compound that gives flavor and aroma to cinnamon (Human Metabolome Database [HMDB], 2017), was reported by Ka (2003) to inhibit tumor cell proliferation, while Imai *et al.* (2002) showed that it decreased adenoma carcinoma formation.

Despite the presence of these angio-suppressive phytochemicals in *C. cebuense* extracts, this property of any endemic Philippine cinnamon species is yet to be studied and explored. Therefore, this study aims to investigate the antiangiogenic effect of *C. cebuense* and its corresponding effect to the morphological development of *A. platyrhynchos* embryo.

Materials and methods

Preparation of the aqueous extract

The leaves of *C. cebuense* were collected from Ramon Aboitiz Foundation Inc. (RAFI) nursery located at Barangay Busay, Cebu City. Permit to collect was issued by RAFI, and plant samples' species identification was verified by the CNU Plant Taxonomy Lab.

After collection, the leaves were washed with running water and air-dried for 3 days. The leaves were cut from its petiole along the midrib, leaving only the blade of the leaves. The blades were then shred and homogenized using an electric blender to obtain fine powder of the plant sample.

Through the conventional process of decoction, five grams of dried and powdered leaves with 250mL triple distilled water were boiled for 45 minutes and cooled to room temperature. The crude extract was obtained and separated from the remaining particles by passing through a Whatman™ No. 1 filter paper.

The crude extract was subjected to serial dilution using normal saline solution [NSS] (0.90% NaCl) to produce five different concentrations: (100%, 50%, 25%, 12.5% and 6.25%).Twenty mL was taken from the crude extract and this served as 100% *C. cebuense extract* (CCE), while 10mL from the 100% CCE was diluted with 10 mL of NSS producing 50% CCE. Next, 10mL was taken from the 50% solution and mixed with another 10mL NSS in order to produce 25% concentration. From the (25%) solution, another 10mL was diluted with 10mL NSS producing the 12.5% concentration. Lastly, 10mL from the 12.5% was diluted with another 10mL NSS to produce the fifth solution which has 6.25% concentration. These five solutions were transferred into individual reagent bottles and were stored at 20°C until use.

Experimental design

The experiment employed a Completely Randomized Design (CRD) with 4 control groups: Positive Control/Pos CON (Retinoic Acid), Vehicle Control (0.90% NaCl), Solvent Control (triple distilled water) and Non Manipulated Control/Non Man CON. There were five experimental groups, as follows: Treatment 1 with 100% CCE, Treatment 2 with 50% CCE, Treatment 3 with 25% CCE, Treatment 4 with 12.5% CCE, and Treatment 5 with 6.25% CCE. There were 3 replicates for each treatment, and each replicate is represented by 4 egg samples. Varying levels of CCE and control groups were randomly introduced to each egg employing a draw by lot method.

Experimental protocol

This study utilized a modified protocol adopted from previous studies employing chorioallantoic membrane (CAM) assay (National Institute of Health [NIH], 2010; West *et al.*,1985; Nambiar *et al.*, 2016; Chen *et al.*, 2013).

One-hundred eighty (180) day-old duck eggs were purchased from a local duck hatchery, soil debris removed using a wet tissue, and placed in an incubator (36.5°C – 37.5°C; 45 – 50% humidity level). The eggs were manually turned upside down daily (12-hour interval) until the 16th day of incubation. On the fourth day, the eggs were candled in order to discard infertile eggs and the air sac location was determined by marking with a pencil.

The administration of the test extracts of C. *cebuense* (100%, 50%, 25%, 12.5% and 6.25%) together with pure grade retinoic acid, 0.90% NaCl, and distilled water was conducted on the 10th day of incubation. Except for the non-manipulated control, individual eggs were holed using a 2mL syringe with 21-G needle at the marked area where the air space is located. Using BD Tuberculin Syringe 27G 1 cc, 0.10mL of each test extract and solution was introduced to the air space region through the hole. After every

inoculation, the egg's pinhole was immediately covered with a micropore tape (3M™) to avoid contamination and eggs were returned to the incubator until the 16th day.

On its 16th day, the CAMs were harvested. Starting at the holed airspace region, the shell from each egg was carefully removed in order not to damage the CAM. When the egg was open halfway, the entire CAM was removed cautiously and mounted immediately onto the labeled Whatman™ No.1 filter paper. The harvested CAMs were air-dried for 1 – 2 min before photo documentation. To ensure consistency of camera distance to the CAM, a 6 in. tall camera tripod was utilized to mount the camera lens (Nikon™ D3400). Meanwhile, embryos from each treatment groups were taken from the yolk and albumin, placed in the petri dish filled with NSS and assessed morphologically. Embryonic morphology was compared to the standard morphological parameters of Hamburger and Hamilton (1951).

Morphometrics and data analysis

The researchers evaluated the embryos' four morphometric measurements (crown to rump length, forelimb length, hindlimb length and beak length) in mm using a Vernier caliper. For all treatment and control groups, data were tabulated only for viable embryos (i.e. all four morphometric parameters are present).

Meanwhile, for CAM measurement, Image J Analysis software (Image J 1.46r), a powerful image analysis program for the quantification of angiogenesis in the duck CAM was utilized. For the quantification of the CAM arteries, the contrast and brightness of the photographed CAMs were adjusted and the protocol followed after Ferreira and Rasband (2012).

The fractal dimension index (D) was calculated by the software after the image was processed. This fractal dimension index is a good statistical descriptor of space-vessel filling area, length and complexity of the capillary networking (Kirchner *et al.*, 1996).

Statistical analysis

For both morphological parameters and fractal dimension index (D), this research employed One-Way Analysis of Variance (ANOVA). Significant ANOVA result was followed by a Post hoc analysis (Tukey's HSD test) at $p \leq 0.05$. All statistical analyses were performed using SPSS version 24. All data were expressed as mean value ± standard error of the mean.

Results and discussion

General observation

From the starting sample size of 180 eggs, a total of 108 fertilized eggs were considered final samples during the experiment. Seventy-four (74) embryos from the one-hundred eight (108) samples were recorded to be alive and all of them exhibited a normal development based on the guidelines of Hamburger and Hamilton (1951) for embryo grading on the 16th day of development.

Table 1. Summary of alive and dead embryos from four control groups and five *C. cebuense* extract (CCE) treated groups.

Groups	Number of samples (n=108)	Number of alive embryos (n=74)	Number of dead embryos (n=34)
Non-manipulated Control	12	12	0
Solvent Control	12	12	0
Vehicle Control	12	12	0
Positive Control	12	0	12
Treatment 1 (100% CCE)	12	3	9
Treatment 2 (50% CCE)	12	9	3
Treatment 3 (25% CCE)	12	9	3
Treatment 4 (12.5% CCE)	12	8	4
Treatment 5 (6.25% CEE)	12	9	3

Table 1 shows 0% mortality rate for Non-manipulated Control (Non Man Con) which is an indication of fair quality among the fertilized egg samples. Solvent control and Vehicle control also show 0% mortality rate. Positive Control (Pos Con) acquired 100% mortality rate, and was expected to inhibit angiogenesis, signifying that decreased density of blood vessels will lead to embryo malformation and death (Herrera *et al.*, 2010). Dead embryos were characterized by foul odor brought by the presence of molds and bacteria, black chorioallantoic membrane, and deformation of the embryos. The number of dead embryos for each treatment group shows a non-linear dose-response trend (Table 1); with T1 (100% CCE) showing the most number of dead embryos (75% mortality rate), followed by T4 (12.5% CCE) with 33.3% mortality, and the rest of the treatments reported at least 25% mortality.

Table 2. Mean fractal indices of photographed CAMs from control and experimental groups. Results are presented as mean±SEM.

Groups	Mean Fractal Index
Non-Manipulated Control	1.28 ± 0.03^{a}
Solvent Control	1.24 ± 0.02^{ab}
Vehicle Control	1.18 ± 0.01^{a}
Positive Control	0.11 ± 0.11^{bc}
Treatment 1	0.30 ± 0.16^{bc}
Treatment 2	0.86 ± 0.19^{a}
Treatment 3	0.99 ± 0.17^{a}
Treatment 4	0.88 ± 0.19^{a}
Treatment 5	0.87 ± 0.19^{a}
ANOVA ($p<0.05$)	.000*

Using ANOVA at ($p \leq 0.05$), * indicates significant difference.
Different letter superscripts within columns indicate significant difference.

The hardness of the egg shell is remarkably decreased under groups T1 (100% CCE) and T2 (50% CCE). This observation may imply that higher concentrations of CCE are capable of softening the hard egg shell of the dead embryos. According to Johnson (2016), environmental issues that cause the softening of the egg shell include toxins (e.g. mold/fungi, bacteria, etc.). Upon observing the dead embryos, molds and bacterial growth are visible inside the eggs (from T1 and T2) and it is most prominent in T1 group which is comparable to the dead embryos from the Pos Con (**Fig. 1**). Earlier findings also associate regional variations of shell pore density and gas conductance with CAM's capacity of gas exchange, which is affected greatly by blood vessel density (Vyboh *et al.*, 2010). Since gas exchange is associated with softened shells especially during hot temperatures (Daniels, 2009), it is inevitable that blood vessel density will be correlated with the softening of shells.

Morphological development

There are four morphological aspects that were measured as indicators of the effect of CCE on the duck egg embryos: crown to rump length (CRL), hindlimb length (HL), forelimb length (FL), and beak length (BL) (**Fig. 2 and 3**). Alive embryos from the treatment groups showed comparable morphogenesis with the embryo from Non Man Con, though differ slightly in some measurements of the aforementioned morphological parameters with respect to its CCE concentration.

As shown in **Fig. 2 and 3,** Non Man Con group exhibit the highest mean across all morphological parameters thus obtained 100% normally developed embryos as described by Hamburger and Hamilton (1951). Solvent control and Vehicle control yielded homogenous results comparable to the Non Man Con group, whereas Pos Con consistently acquired the

lowest mean for all the parameters. This result is in accordance with Cohen and Shiota's (2002) claim that retinoic acid at elevated dosage can induce teratogenesis and lead to abnormal structural development, such as craniofacial and limb abnormalities in humans and ocular anomalies in mice (Gupta, 2017).

Fig. 1.Partially opened eggs (at day 16), showing dead embryos from Treatment 1 (100% CCE [A]) and PosCon (Retinoic Acid [B]).

Meanwhile, morphometric analysis of the embryos from the experimental groups revealed a non-linear dose-response trend for CRL, HL, FL, and BL except for Treatment 1 (100% CCE) that greatly stunted all its morphological development evident in its low means of 15.00mm, 7.50mm, 5.00mm, and 2.50mm, respectively (Fig. 2 and 3). It is an indication that T1 offers an optimal concentration that could inhibit morphological growth. Treatments 2 (50% CCE), 4 (12.5% CCE), and 5 (6.25% CCE) yielded comparable results, with slight or no difference at all.

The observed fluctuation in the morphological growths of these three treated groups indicates no direct relationship between the morphological changes and the changes in concentration of the extract. Treatment 3 (25% CCE), however, exhibits the highest morphological means of 44.58mm (CRL), 21.25mm (HL), 17.50mm (FL), and 7.50mm (BL). This data suggests that T3 offers the most favorable concentration that could support normal morphogenesis of the embryo comparable to Non Man Con, Vehicle Control, and Solvent Control.

Angiogenesis activity

Analyzing the Mean Fractal Index (MFI) of the photographed chorioallantoic membranes (CAMs) from duck eggs among control and experimental groups (Table 2) allows the quantification of their angiogenesis. High values of MFI indicate complex branching points of blood vessels, and hence induced angiogenesis; whereas low MFI values suggest inhibition of the blood vessel formation. As expected, Pos Con yielded the lowest MFI which corresponds to claims that retinoic acid has anti-proliferative effect against tumors through its inhibition of angiogenesis (Hoffmann *et al.*, 2007). Meanwhile, negative control groups (i.e. Non Man Con, Solvent Control, and Vehicle Control) yielded high and comparable MFI results (Table 3).

CAMs treated with *C. cebuense* extract (CCE) poses observable effects in its branching complexity with respect to its administered concentration. The notable effects of CCE on CAMs comparable to the responses in Pos Con imply the presence of antiangiogenic constituents in *C. cebuense*.

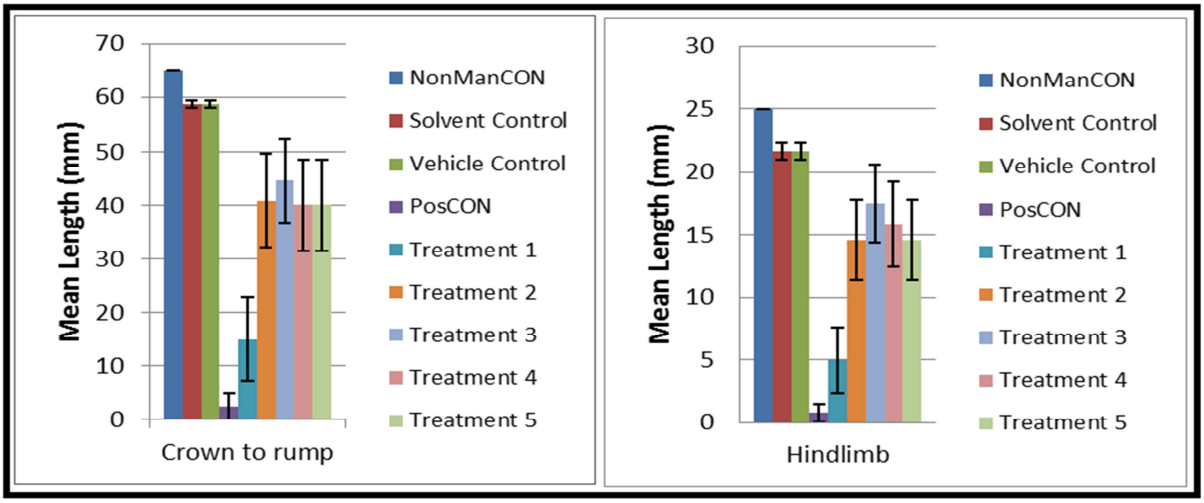

Fig. 2a – 2b. Crown to rump length (Fig 2a.) and hindlimb (Fig 2b.) parameters of alive embryos from different control and treatment groups. Results are presented as mean ± SEM (with error bars). Solvent Control (Triple Distilled Water); Vehicle Control (Normal Saline Solution [0.9% NaCl]); Positive Control (Retinoic Acid); Treatment 1 = 100% CCE; Treatment 2 = 50% CCE; Treatment 3 = 25% CCE; Treatment 4 = 12.5% CCE; Treatment 5= 6.25% CCE.

This angiogenesis suppression can be attributed to the presence of bioactive compounds such as terpene and terpenoid groups that have potential antiangiogenic activity by reducing vascular endothelial growth factor (VEGF) expression (Bento *et al.*, 2009; Ragasa *et al.*, 2013).

Among the experimental groups, the lowest MFI of 0.30425 was manifested by T1 (100% CCE); while T3 (25% CCE) exhibited the highest MFI suggesting a capacity to induce vascular development in the CAM **(Table 2).**

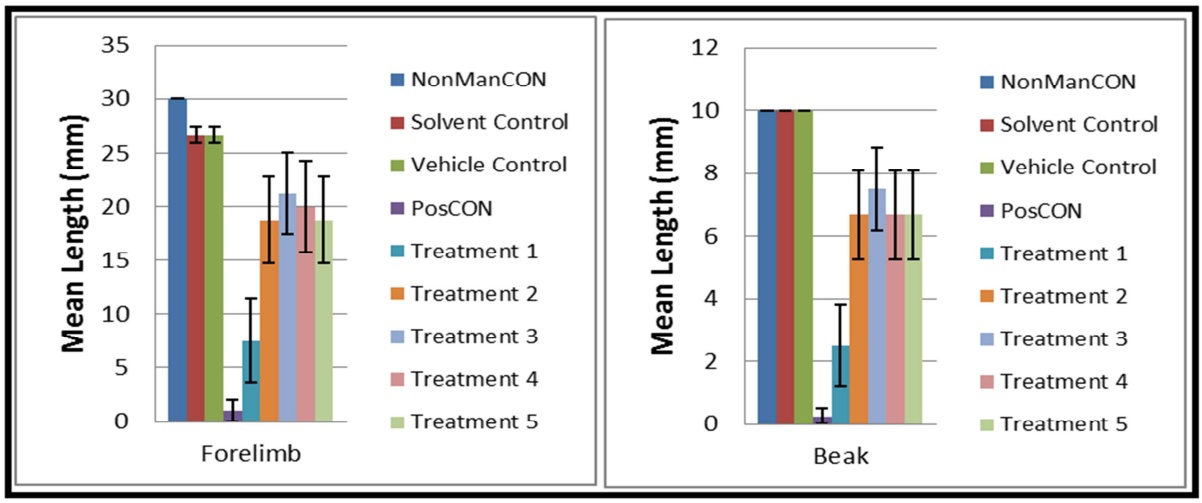

Fig. 3a – 3b. Forelimb (Fig 3a.) and beak length (Fig 3b.) parameters of alive embryos from different control and treatment groups. Results are presented as mean ± SEM (with error bars).

Treatment 1 therefore strongly reduced branching complexity and proliferation of the blood vessels, entailing the optimal potential of angiogenesis inhibition among others. Other treatment groups (i.e. T2, T4, and T5), however, manifested comparable results in angiogenesis suppression.

Comparative analysis of the Mean Fractal Indices (MFI) elucidated that CCE treatments indeed

influenced the suppression of blood vessels' formation in ducks' CAM. Results **(Table 2)** revealed statistically significant difference (p=0.000) between five experimental groups and four control groups. A non-linear dose-response relationship between the MFI and various treatments was also established based on the results.

Finally, the results in this study corroborate to previous studies that cinnamon species indeed share phytochemicals that can inhibit angiogenesis. In fact, a recent study by Lorion *et al.* (2016) showed that another Philippine endemic species, *C. mindanaense* also inhibit angiogenesis and morphogenesis at its most potent concentration of 100% owing to the bioactivity of its compounds. Meanwhile, *C. cebuense* extract exhibited significant results on the morphological development of the embryos and angiogenesis in the chorioallantoic membranes.

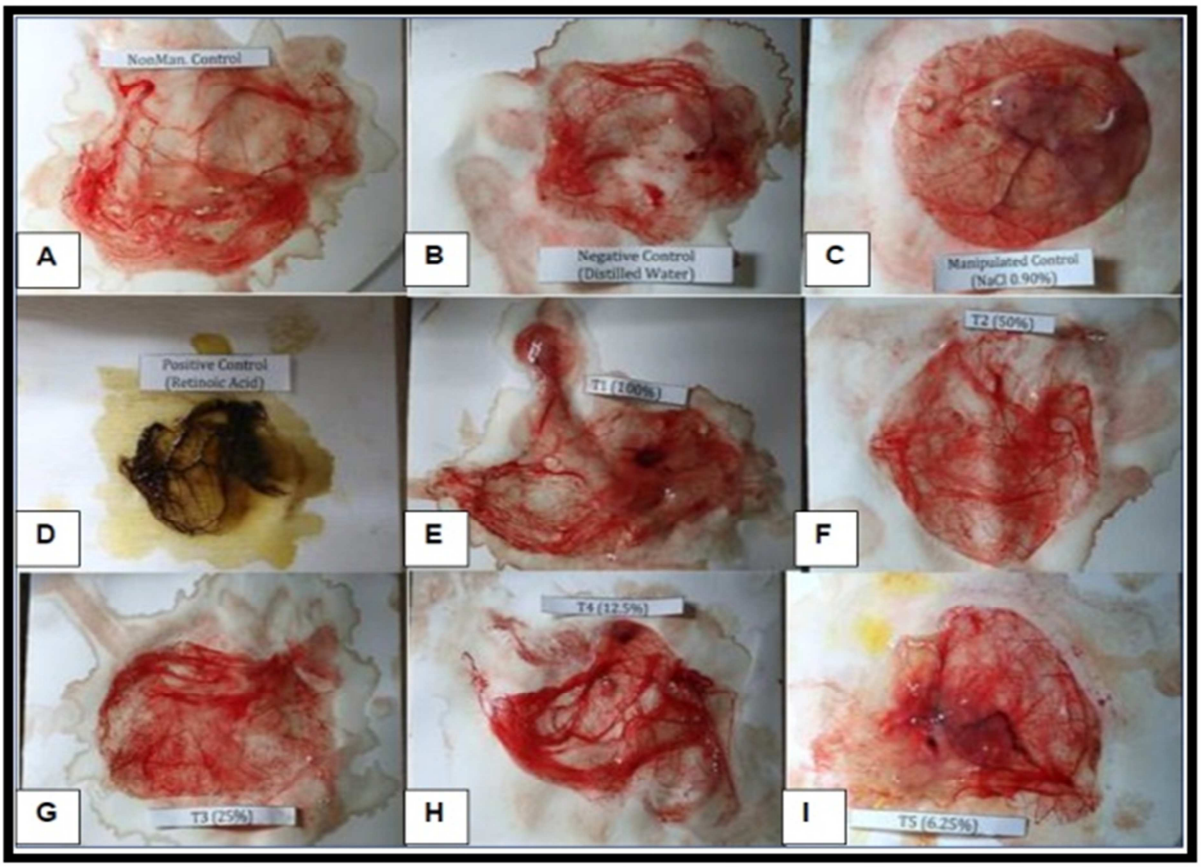

Fig. 4. A-I. Photographs of the different chorionallantoic membranes (CAMs) of duck embryo on Whattman no.1 filter paper from the control and treatment groups: [A] Non Man Con; [B] Solvent Control = Triple Distilled Water; [C] Vehicle Control = Normal Saline Solution (0.9% NaCL); [D] Positive Control = Retinoic Acid; [E] Treatment 1 = 100% CCE; [F] Treatment 2 = 50% CCE; [G] Treatment 3 = 25% CCE; [H] Treatment 4 = 12.5% CCE; [I] Treatment 5= 6.25% CCE.

The results helped establish a direct relationship between angiogenesis activity and embryonic development of ducks wherein suppression of angiogenesis can lead to abnormal embryonic development while stimulation of angiogenesis can promote morphogenesis. This relationship finds support from Irvin *et al.* (2014), Sagal *et al.* (2006), and Penn (2008) who claimed that regulated angiogenesis play a critical role in normal embryonic development, whereas dysregulation of the process can be observed in a wide array of diseases.

Based on the results, the researchers concluded that 25% of *C. cebuense* extract is the most effective

concentration that could stimulate the vascularization of blood vessels that transport oxygen and nutrients, and hence promote embryonic development. Meanwhile, 100% *C. cebuense* extract has the most potential concentration for angiogenesis inhibition to occur, and thus, could be used for therapeutic approach against cancer. Future research directions can focus on utilizing the bark, fruit, flower or roots of *C. cebuense* utilizing appropriate solvents and fractions; as well as in utilizing *C. cebuense* and other Philippine cinnamon species for synergistic antiangiogenic effects.

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