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Evaluation of antiangiogenic property of *Ocimum basilica* ethanolic leaf extract by using duck embryo chorioallantoic membrane (cam) assay and its morphometric analysis

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Abstract
Angiogenesis is the formation of new vessels that sprouts from endothelium, where there is endothelial cell migration, tube formation and proliferation. The *Ocimum basilica*, is an important herb under the Family *Lamiaceae* housing the largest number of medicinal species known for its therapeutic significance. The setups were composed of a vitamin A as positive control, 90% ethanol as negative control, *O. Basilica* ethanolic leaf extract in 1 mg/mL, 3 mg/mL and 5 mg/mL concentrations. The different concentrations were applied on the 8th day of incubation. The antiangiogenic effect of *Ocimum basilica* by taking the average number of branch points using CAM and was determined after two days. Results shows that angiogenesis was induced in the negative control while the inhibition of angiogenesis was significantly reduced with the *O. basilica* ethanolic leaf extracts. It also showed that the greater the dosage, the lesser the branched points observed. Thus, *O. basilica* ethanolic leaf extract might have a very promising antiangiogenic potential.

Keywords: Angiogenesis, chorioallantoic membrane, endothelium, *Ocimum basilica*

1. Introduction
Chorioallantoic membrane (CAM) assays have been greatly used to study angiogenesis, metastasis and tumor cell invasion [1]. Hertig first named the word angiogenesis in 1935, and Folkman revealed the mechanism in studying tumor angiogenesis [2].
The chick or duck embryo chorioallantoic membrane (CAM) model is an extra-embryonic membrane that is knowingly used *in vivo* to study either angiogenesis or anti-angiogenesis or both. An angiogenic feedback or response occurs seventy-two to ninety-six hours (72–96 hr) after stimulation in the condition of increased vessel density around the implant, with the blood vessels radially converging onto the center like spokes in a wheel [3]. In contrary, when an angiostatic compound is applied, the vessels become less dense on the implant where it is applied and even disappear [4]. Quantitation of vessels in a lot of CAM models can be utilized to screen drugs from organic samples like plant extracts [5]. The increasing interest in anti-angiogenic therapy for tumors and cancer requires the advancement of a quantitative angiogenesis assay. An example of a useful *in vivo* process that has been used broadly in angiogenesis research is the high vascularization of blood vessels in chick embryo CAM of the chicken embryo [6]. The CAM assay has become an extensively used tool for the determination of angiogenesis property and anti-angiogenic property of various drugs including herbal extracts. The accomplishment of anti-angiogenic therapy for treating cancer has led to an explosion in the field of research for potential anti-angiogenic agents [1].
Now, many *in vivo* angiogenic assays have been advanced to investigate angiogenesis in pathological and physiological circumstances and both pro- and anti-angiogenic effects of any compound [7]. The chick embryo CAM developed as a sensitive, successful, and feasible model for an *in vivo* research on both angiogenesis and anti-angiogenesis. In the management of tumor and cancer patients, many drugs are applied together in clinical practice, thus rich in antioxidant materials could lessen these abnormal, proliferative cells [3].
Most of Filipinos composed of 68.4% from the rural areas and 51.5% from the urban areas commonly use Complementary and Alternative Medicine to relieve their medical conditions.

Because of the popularity of plants as Complementary and Alternative Medicine modality, many herbal preparations such as infusion, also known as tea, became available in markets [8]. *Ocimum basilica*, locally known as Sangig, belongs to the family Lamiaceae, exhibiting a pleasant smell growing in several regions with temperate regions all over the world [9]. The family Lamiaceae known as the mint family houses a large number of medicinal species [8]. *Ocimum basilica* is a common herb known for both its ornamental and therapeutic significance. The plant was reported to contain chemical constituents such as terpenoids, alkaloids, tannins, flavonoids, ascorbic acid and saponin glycoside [10]. On the other hand, it is known to be hepatoprotective, antihyperglycemic, antitoxic, anti-inflammatory, anti-fungal, anti-bacterial, and hypolidemic [10].

2. Materials and Methods

The materials used in the said activity are as the following:
Medium for CAM Assay: Eight-day Duck Egg Embryo
Plant Material: Ocimum basilica, locally known as "Sangig," from the family of Arecaceae
Apparatuses: Dissecting needle, Stereomicroscope, Egg tray, Parafilm, Light for Candling, Gloves, Mask, Experiment, Pencil for labels, Incubator, Petri dishes/ Petri plates, Vernier Caliper, and 10% Formalin

2.1 Plant Extraction

The leaves of *Ocimum basilica* was collected from Caraga State University, Ampayon, Butuan City. The collected leaves were air-dried for one (1) week. The dried leaves were powdered mechanically by a sterile blender and stored in air tight container. The fine, dried powdered 30 grams (30g) of the dried leaves was macerated in 300mL of aqeous ethanol ethanol for forty-eight (48) hours at room temperature with intermittent shaking. The extracts were filtered through an ordinary filter paper and were concentrated at 60°C using a rotary evaporator and were placed in a water bath until crude extract is produced [11].

2.2 Preparation of Duck Eggs

Eight-day *Anas platyrhynchos*, Duck, Eggs was bought at Basag, Ampayon, Butuan City. Eggs were cleaned with the aid of 70% ethanol to remove dirt and other debris that could infect the eggs when opened. All the eggs that were used were incubated at 37 ^{0}C, with about humidity of 65.5 %. Three fertile 8-day duck eggs were used for each: positive control, negative control, and the treatment, 1, 3, and 5 mg/mL (as determined by LD50 through a source [12]).

2.3 Assay Proper

The method that was used is adapted from Ribatti. Sterile conditions and labelling were properly maintained.
Candling experiment was done prior to treatment to check for the viability of the eggs. A flashlight was placed under the eggs to view the position of the developing embryo. Those eggs with underdeveloped and even dead embryos were discarded. The concentrated extract was assigned to 1, 3, and 5 mg/mL concentrations as the experimental group. A 90% ethanol was used as a negative control and retin, as a source of retinoic acid, Vitamin A, was used as positive control. A window in the egg shell about 1x1 cm was made to expose the Chorioallantoic membrane to pierce for experimental manipulation. About 100 µl of the extract, positive and negative control were placed onto the CAM. The treated eggs were sealed with parafilm and the eggs were then incubated for two days at 37 ^{o}C and 65.5%. humidity. Triplicate was done. Between day 8 and day 10, the growing CAM vasculature is ready to grow in response to additional proangiogenic stimuli, and it is very responsive to antiangiogenic factors and those are the reason why day 8 is the subject for experimental treatment. On the 10th day of incubation, the CAMs will be harvested by removing the hard shell withdrawing intact the soft membrane covering the embryo [13].
The Chorioallantoic membranes at the site of application for were examined. Quantitation was performed 2 days after implantation and involved counting the number of CAM vessels in the area under the stereomicroscope. The newly formed blood vessels come out converging toward the disk in a wheel-spoke pattern in response to proangiogenic stimuli. Inhibition of angiogenesis by antiangiogenic compounds results in the lack of new blood vessel formation and sometimes in the disappearance of pre-existing vessel networks. Four quadrants of the CAM in the area were drawn. The blood vessel branch points at each area of the different quadrant were counted manually in a clockwise direction[13].

2.4 Morphometric Analysis

The embryos that will be isolated from the experiment will be weighed by a digital balance to have the data with their weights. The morphometry of every embryo that was used for the experiment will be measured using a vernier caliper. The following indices will be measured: (1) Crown-rump- length (CRL), the measurement from the crown, the skull vertex, to the midpoint between the rump or the apices of the buttocks ; (2) Head beak length (HBL), the measurement from the back of the head of the embryo to the tip of the beak, (3) forelimb length (FL), the measurement between where the forelimb is connected and the tip of the forelimbs and, (4) hind limb length (HL), the measurement between where the hind limb is connected and the tip of the recognizable hind limb.

2.5 Statistical Analysis

Comparison between groups was made by One-Way Analysis of Variance for the vascular densities and morphometry by IBS SPSS v.20. Differences with $P<0.05$ between experimental groups were considered statistically significant.

3. Results and Discussions

3.1 Plant Extraction

A stock solution of 6.8 mg/mL was produced. Then a concentration of 1, 3, and 5 mg/mL was then prepared.

3.2 Preparation of Duck Eggs

Eggs were cleaned with the aid of 70% ethanol and were incubated 37 ^{0}C, with about humidity of 65.5%.

3.3 Proper Assay

All the eggs were viable for the treatment as the candling experiment was done to all.
Gross morphologic observations of the Chorioallantoic membrane harvested from the samples that were treated showed many pathologies which have corresponded with the vascular densities of the treated samples. Part of vascular pathology that was observed were disrupted the growth of chorioallantoic capillaries and had irregularly branched capillaries and thin veins. In few pathologic samples, there was a distinct obstruction in the growth of new vasculature in the major veins which could be linked to the treatment and the mortality of the samples. In comparison, the CAM arteriolar

endothelium from the negative control, which is water, displays a more extensive junctional complex with multiple membrane contact points. With the positive control, less vascularization is observed.

The inhibition of angiogenesis was significantly reduced with the *O. basilica* ethanolic leaf extracts. It was supported by the statistical analysis that there was a substantial difference on the antiangiogenic effect of *O. basilica* ethanolic leaf extract using Chorioallantoic membrane assay on the vascularization of the embryos. It also showed that the greater the dosage, the lesser the branched points observed. (Figure 2 and 3)

The results of the CAM assay have shown that the *Ocimum basilica* extracts were significantly different ($P<0.05$) from the negative control.

3.4 Morphometry Analysis

The weights of the duck embryos were significantly different to each other ($P<0.05$). The duck has smaller weight in higher concentration. (Figure 4)

The duck embryos that were treated by the *Ocimum basilica* extract were clearly observed as small compared to other treatments, including its crown-rump length, head-beak length, fore limb length, and hind limb length. The higher the concentration, the smaller the duck. (Table 1, Figure 1, 4 and 5)

4. Conclusion

The CAM assay administers a suitable model to test the effects of angiogenic or anti-angiogenic agents coming from an organic sample. However, the quantification of the effects is not easy for all. Counting large vessels method is based on optical and visual examination, even manual vessel counts or distribution of vascular network, global measurements of the spatial pattern, is usually used.

It was supported by the statistical analysis that there was a substantial difference on the antiangiogenic effect of *O. basilica* ethanolic leaf extract using Chorioallantoic membrane assay on the vascularization of the embryos. It also showed that the greater the dosage, the lesser the branched points observed. Thus, these findings of the study indicated that *O. basilica* ethanolic leaf extract might have a very promising antiangiogenic potential.

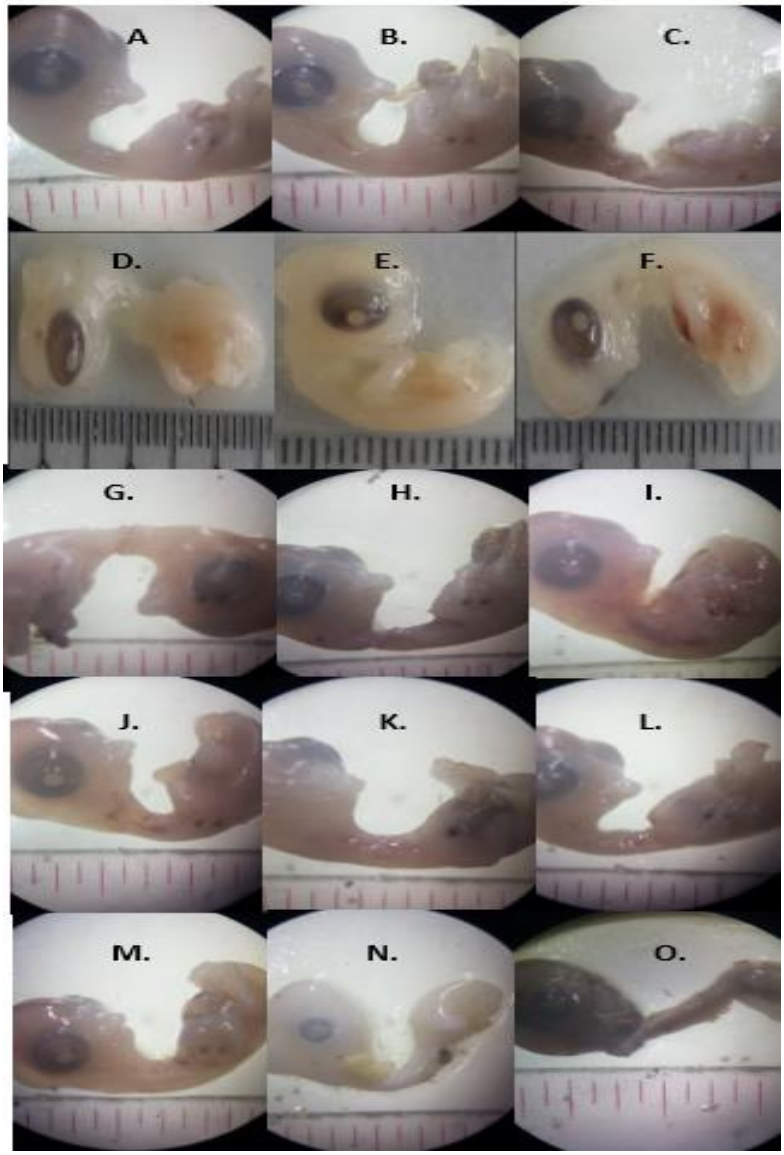

Fig 1: Duck embryo of different treatments. A-C. Duck embryos that were treated by negative control show large measurement of morphometry. D-F. Duck embryos that were treated by positive control show medium measurement of morphometry. G-I. Duck embryos that were treated with 1 mg/mL *Ocimum basilica* show medium measurement of morphometry. J-L. Duck embryos that were treated with 3 mg/mL *Ocimum basilica* show medium measurement of morphometry and some malformations. M-O. Duck embryos that were treated with 5 mg/mL *Ocimum basilica* show very small measurement of morphometry and malformations.

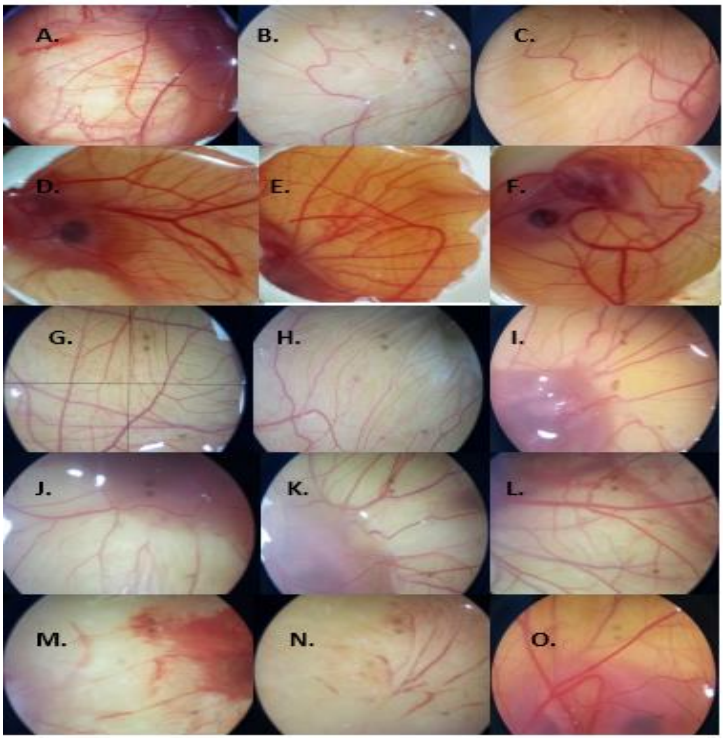

Fig 2: Blood vessels of different treatments. **A-C.** Duck eggs that were treated by negative control shows very high branching points. **D-F.** Duck eggs that were treated positive control shows very low branching points. **G-I.** Duck eggs that were treated with 1 mg/mL *Ocimum basilica* extracts shows high branching points. **J-L**. Duck eggs that were treated with 3 mg/mL *Ocimum basilica* extracts shows slightly low branching points. **M-O.** Duck eggs that were treated with 5 mg/mL *Ocimum basilica* extracts shows almost no branching points.

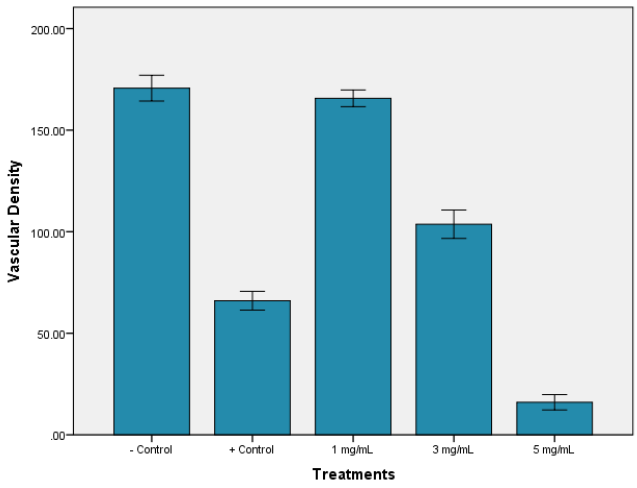

Fig 3: Vascular Densities of different treatments at P<0.05. The angiogenesis was induce in the negative control while the angiogenesis inhibition had been strongly reduced upon the treatment of *Ocimum basilica* ethanolic leaf extract. Results are presented as mean ±SEM

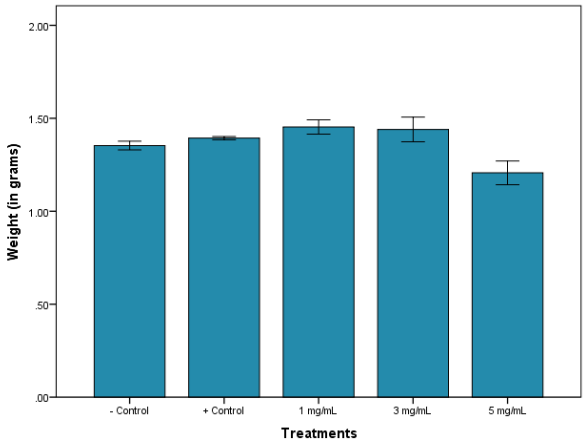

Fig 4: The overall corresponding weight of embryos of different treatments at P<0.05. In negative control, the embryo slightly weighed less compared to the application of Retinoic acid (Positive control). It also shows that the greater the dosage of Ocimum basilica ethanolic leaf extract the lesser the weight of the embryos in grams. Results are presented as mean ±SEM.

Table 1: Summary of the morphometry of different treatments with its mean±std.error. Superscript *a* is an indication that it is significantly different from each other (P>0.05)

Treatment	BW (g)	CRL (mm)	HBL (mm)	FLL (mm)	HLL (mm)
- Control	1.353±0.023[a]	20.666±0.333[a]	10.000±0.577[a]	4.333±0.333[a]	5.666±0.333[a]
+ Control	1.393±0.008[a]	19.000±1.527[a]	9.666±0.333[a]	4.166±0.726[a]	4.000±0.577[a]
1 mg/mL	1.453±0.038[a]	22.333±0.666[a]	10.666±0.333[a]	4.333±0.333[a]	6.333±0.333[a]
3 mg/mL	1.440±0.066[a]	22.666±1.763[a]	9.333±0.333[a]	4.666±0.333[a]	6.333±0.333[a]
5 mg/mL	1.206±0.063[a]	18.000±1.527[a]	8.333±0.666[a]	2.333±0.881[a]	4.000±0.577[a]

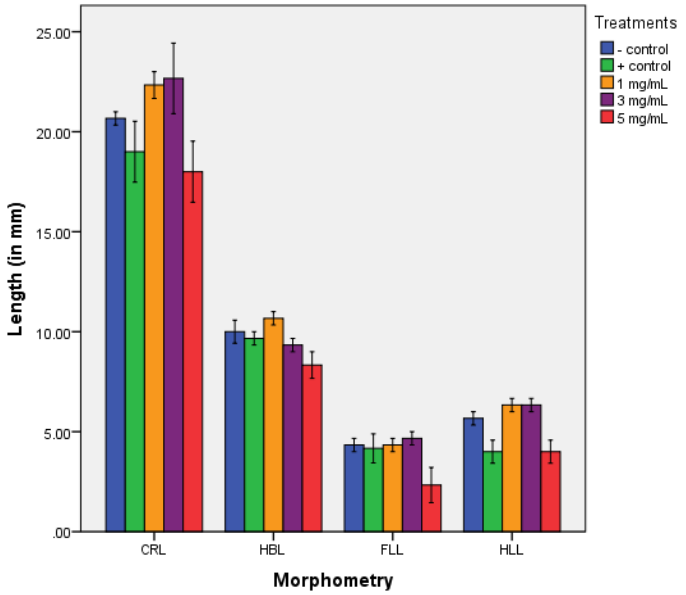

Fig 5: Morphometry in millimeters of the embryos with the different treatments at p<0.05. Negative control; CRL-Crown rump length; HBL-Head beak length; FL-forelimb length; HL-Hindlimb length. Results are presented as mean ±SEM.

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