A Pilot Investigation on the Potential Bioactivity of Calabash (*Crescentia Cujete*) Fruit Extract in the Angiogenesis and Morphometry of Duck (*Anas Platyrhynchos*) Embryo

Paul John B. Pastor and Florence Jhun F. Almadin

Abstract**—** The potential bioactivity of the methanolic fruit extract of calabash (*Crescentia cujet*e) to the angiogenesis and embryonic development of duck (*Anas platyrhynchos*) embryo was assessed in this study. Chorioallantoic membrane assay and morphometric analysis were conducted. Concentrations of 25%, 50%, 75% and 100% were obtained as test samples and distilled water was used as the control variable. Results showed that the extract under the concentrations of 0.12 g/mL, 0.24 g/mL, 0.35 g/mL and 0.47 g/mL (25%, 50%, 75% and 100% respectively) was able to significantly target the blood vessels, leaving only few branch points to measure. The 75% and 100% concentrations also exhibited mostly zero development of the embryos, leaving low morphometric results which is also significantly different from the control group ($p<0.05$). In comparison between treatments, morphometric analysis under the head-beak length measurement also showed significant differences in between the 25% and 100% concentration. Hence, results suggest that *C. cujete* is anti-angiogenic and therefore essential for further intensive study on tumor angiogenesis and metastasis. However, it is highly toxic due to observable underdeveloped morphology and mortality of duck embryos at higher concentrations.

Keywords**—** *Anas platyrhynchos*, chorioallantoic membrane assay, *Crescentia cujete,* angiogenesis, morphometry

I. Introduction

Angiogenesis or the formation of new blood vessels from pre-existing blood vessels, is a mechanism that is essential during embryonic development, menstrual cycle and in wound healing [1][2][3]. However, it also plays a great part in pathology which contributes in diseases like cancer [4].

This study aims to determine the potential bioactivity of the methanolic fruit extract of the Calabash (Crescentia cujete) on the angiogenesis of the duck (Anas platyrhynchos) chorioallantoic membrane. The study also seeks to determine if the angiogenic process is enhanced by the bioactivity of the methanolic extract of the calabash fruit, or if an inhibitory effect is displayed. Furthermore, the study determines its effects on the morphometric indices of the duck embryo after exposure to the CAM assay.

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II. Materials and Methods

The Calabash fruit used in the study was collected in Butuan City, Philippines. The collected plant specimen was identified by the Department of Environment and Natural Resources (DENR). Plant samples were deposited at the Father Saturnino Urios University Biology Laboratory.

Following the method of Almadin et al., [5], the pulps of the fruit were air dried for a week. The dried pulps were weighed by an analytical balance and methanolic extraction was done by using 90% methanol. Air dried materials were grounded and weighed for 200g, stored in an Erlenmeyer flask and was treated with 220 ml of 90% methanol. The mixture was filtered with Whatman filter paper. The filtrate of the plant extract was concentrated over an electric heater at temperature below 50˚C and reduced to 23 mL, leaving minimal traces of alcohol behind [5]. The concentrated extract was then divided into 4 concentrations of 0.12 g/mL, 0.24 g/mL, 0.35 g/mL and 0.47 g/mL each representing 25%, 50%, 75% and 100% respectively.

Eight-day old Anas platyrhynchos developing eggs were used and acclimated for about 24 hours. Acclimated eggs were taken out of the incubator for treatment. Egg candling was done prior to treatment to check for viability. Eggs were placed in front of a flashlight to view the position of the developing embryo. Those with underdeveloped embryos were discarded [6]. The eggs were selectively randomized and a 1cm x 1cm opening or window was cut onto the surface of the egg over the embryo to expose the chorioallantoic membrane (CAM). Thirty microliters of each treatment was topically applied on the surface of chorioallantoic membrane using a 1mL disposable insulin syringe. The egg window was sealed with disinfected tape, properly labeled. The eggs were incubated for 2 days. Day 9 was chosen for experimental treatment because between day 8 and day 10, the developing CAM vasculature is ready to sprout in response to additional proangiogenic stimuli, and in turn, is very responsive to antiangiogenic factors [7].

TABLE I SUMMARY OF PARAMETERS MEASURED IN THE DUCK EMBRYOS TREATED WITH *CRESCENTIA CUJETE*.

Treatment	% Death	BW (g)	CRL (cm)	HL (cm)	FL (cm)	HBL (cm)
Control (Water)	0	1.321 ±0.04061	35.08±0.9000	10.50±0.4174	9.083±0.6332	13.33±0.5270
25% (0.12 g/mL)	50	0.7392±0.2245	17.75±5.361*	4.750±1.750*	4.583±1.433*	9.333±2.039**
50% (0.24 g/mL)	50	0.6592±0.1994	16.58±5.013*	5.333±1.635*	4.667±1.421*	7.083±2.144
75% (0.35 g/mL)	75	0.3992±0.2201*	8.250±4.321*	2.250±1.194*	1.667±0.8819*	3.500±1.840*
100% (0.47 g/mL)	83.33	0.2183±0.1475*	5.833±3.933*	1.417±0.9571*	1.250±0.8972*	2.250±1.518*

*Results are presented as mean ± SEM. Means with * are significantly to control and ** significant to 100% ($p<0.05$)*

After 2 days of incubation and administration, all duck eggs were removed from the incubator and vascularization of the CAM were examined and captured using a Canon 1100D DSLR. The images were transferred onto a computer and have undergone editing process using the Picasa photo viewer (Picasa, v3.0), to adjust lighting for better, and more detailed view of the blood vessels. The images were transported on Paint Program (Microsoft, 2007) to clearly count all the blood vessel branch points found in the chorioallantoic membrane.

Duck embryos from the CAM experiment were further subjected to morphometric examinations. Morphometry of individual duck embryos were measured using a ruler. The following indices were measured: Crown-rump-length (CRL) as the measurement from the skull vertex (crown) to the midpoint between the apices of the buttocks (rump); Head-beak length (HBL) as the measurement from the back of the head to the tip of the recognizable beak; forelimb length (FL) as the measurement between the top and the tip of the forelimbs and hind limb (HL). Photographs were taken from the entire length of the embryos using the Canon 1100D DSLR. Body weights of every embryo were also measured.

Data was described as means ± SEM. Parameters in percentages (%) mortality rate was also determined. Analysis of variance (ANOVA) was utilized to determine differences between treatment groups for values such as, fetal size, fetal weights, morphometric measurements values, and vascular branch points. When the ANOVA revealed significant differences among treatment groups, Duncan's multiple range test (DMRT) was utilized to pinpoint specific treatment differences. Differences were considered statistically significant if< 0.05 using Graphpad Prism® version 6.0 software.

III. RESULTS AND DISCUSSION

Results

A. Duck Chorioallantoic Membrane (CAM) Assay

The experiment was terminated on the 11th day of development of the duck (*Anas platyrhyncos*) embryo (2nd day post treatment). Chorioallantoic membrane (CAM) was observed for the counting of blood vessel branch points treated with calabash fruit extract. There were a total of 60 eggs used for the assay and four different concentrations of the calabash methanolic extract (25%, 50%, 75% and 100%) were used as treatment groups, each having triplicates of 4 per treatment. The same replication was also done for the control group (distilled water).

Morphological comparison of the vasculature and branch points of the chorioallantoic membrane between control and treatment groups are shown in figure 1. Control group shows multiple and a more detailed branch points marked with meshed string-like structures in the arteriolar section of its chorioallantoic membrane (Fig 1E). Apparent less and poorly developed branch points (Fig 1A-25% and Fig 1B-50%), and absence of vascular growth (Fig 1C-75% and 1D-100%) were mostly observed within treatment groups.

The difference on the morphology of the vasculature and membrane branch points between control and the different groups were significant ($p<0.05$) at all concentrations (Fig 2). There were reduced blood vessel branch points of the chorioallantoic membranes in all groups which half of the samples of 25% and 50%, and most of the samples of 75% and 100% showed zero vasculature.

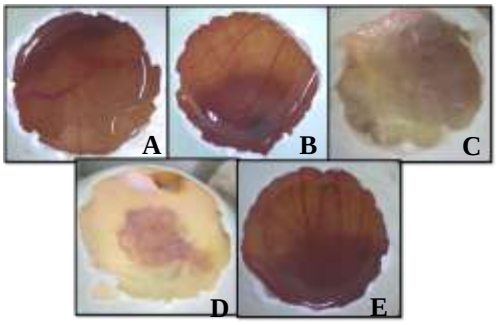

Fig 1 Images showing the effect of C. cujete fruit extract on the CAM vasculature of 11-day old duck embryos. (A.) CAM of embryos treated with 25% (0.12 g/mL) extract; (B.) CAM of embryos treated with 50% (0.24 g/mL) extract; (C.) CAM of embryos treated with 75% (0.35 g/mL) extract; (D.) CAM of embryos treated with 100% (0.47 g/mL) extract; (E.) CAM of embryos treated with the control.

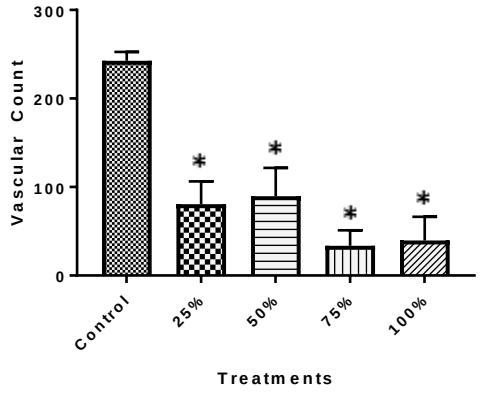

Fig 2 Evaluation of the effect of *Crescentia cujete* extract on the chorioallantoic membrane (CAM) of the 11 day old duck embryo. *-significant at p<0.05.

B. Morphometric indices of duck embryo

Table 1 shows the summary of all indices measured from the embryos. Eggs treated with 75% and 100% concentrations have significantly higher underdeveloped embryos than the rest. Gross pathological observations show that the embryos treated with 75% and 100% extract have the highest embryonic anomaly, leaving significant results in all indices in terms of head-beak length (HBL), crown-rump length (CRL), hind limb length (HL), forelimb length (FL) and body weight (BW). Twenty-five percent and 50% concentrations also showed significant differences to the control under the measurement of crown-rump, hind limb and forelimb (p<0.05), most likely as the result of the treatment.

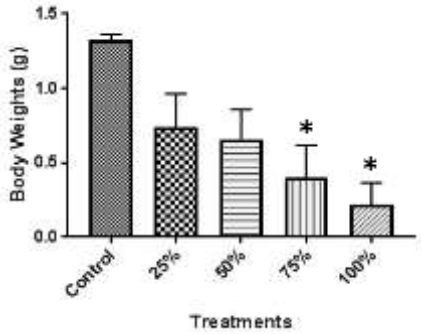

Fig 3 Embryo weight (g) of A. platyrhynchos during the termination of the experiment. *-significant to control at p<0.05

Fig 4 Eleven day old *A. platyrhynchos* embryos treated with various concentrations of *Crescentia cujete*. *(*A,) Control; (B) Embryo treated with 25% (0.12 g/mL) of *C. cujete*. (C) Embryo treated with 50% (0.24 g/mL); (D) Embryo treated with 75% (0.35 g/mL); (E) Embryo treated with 100% (0.47 g/mL) of *C. cujete.*

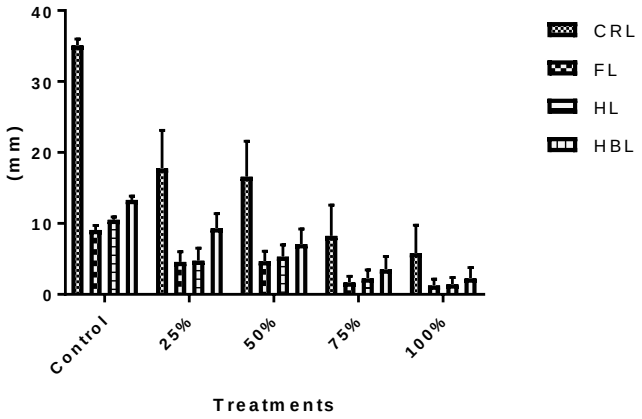

Fig 5 Morphometric indices of the 11-day old duck embryo after treatment with *C. cujete*. (CRL-crown rump length; HBL head beak length; FL-forelimb length; HL-hind limb length.)

The majority of the samples from the 75% (Figure 4D) and 100% (Figure 4E) group has obviously no morphological indices to be quantified, in contrast to the 25% (Figure 4B), 50% (Figure 4C) and to the control (Figure 4A). The total evaluated morphometric measurements (Figure 5) reveal that there is a consistency and significant difference of the control to the four treatments under the evaluation of crown-rump, hind limb and forelimb length; in which poor embryonic development was very evident (p<0.05). The morphometric analysis also showed that the measurement of the head-beak length in the 75% and 100% concentration is significantly different to the control. The head-beak measurement between 100% and 25% concentration is significantly different from each other as well.

Discussion

The results showed by the study may have been caused by the recorded phytochemical constituents of the calabash fruit that were done by with the same collected samples and under the same supervision of this study. The plant is considered to have alkaloids, saponins, flavonoids, tannins and phenols [8]. Even though phytochemicals are beneficial, plant alkaloids, tannins and phenols are also considered to be toxic at a definite level and considered to be corrosive [9]. Chandra Sekhar J [9] also stated that the degree of toxicity also depends on the location (including height above sea level), climatic factors including the local microclimate (light, warmth, and humidity), growing season, soil type, fertilization, plant variety and age. Also, the condition of the poisonous plant material is equally important (dried, chewed, cooked, as tea) and the dose of course is the most important factor.

A. CAM Assay

The CAM has long been considered as favorable system for the study of tumor angiogenesis and metastasis [10][6], because at this stage, the embryo immunocompetence system is not fully developed and the conditions for rejections have not been established[11]. In fact, the immunocompetence in birds develops only after hatching [11]. Using the duck

chorioallantoic membrane for the evaluation of the bioactivity of the methanolic fruit extract of *C. cujete*, it revealed evidences that the fruit extract may contain active compounds that are able to inhibit angiogenesis. *C. cujete* extracts under all the treatments of 24%, 50%, 75% and 100% were able to significantly reduce Cam vasculature. Reduced Cam were more pronounced in the two higher concentrations (75% and 100%), showing mostly zero vasculature. But, with the observed mortality in all the higher concentrations, it is suggested that the use of concentrations be improved and would have to be lowered; so to show less mortality of the samples.

B. Embryonic Development

The concentrations made by the study were able to obtain different result patterns and morphometric indices of the *Anas platyrhynchos* embryo. A large number of observable underdeveloped eggs were shown in the study, making all the concentrations significant for showing mostly no embryonic development since the treatment. Hence, the study was accompanied with mostly zero weights and zero morphometric indices. It is important to note that the highest concentration also showed the highest number of underdeveloped embryos.

IV. Conclusion and Recommendation

The results indicate potential anti-angiogenic property of the Calabash fruit, which is essential for studying tumor angiogenesis and metastasis, but is highly toxic to embryonic development at higher concentrations due to the observed underdeveloped morphology and mortality when statistically compared to the control.

It is recommended that further intensive studies must be done with regards to the potential anti-angiogenic properties of the *C. cujete* fruit. Researches that comprise data with smaller margin of error are encouraged in pursuance of the initial observations brought by the study; which means pursuing even larger sample sizes for secondary examinations of the plant's anti-angiogenic capacity. More novel methods are highly advised to use in order to fully assess the specific properties that enable the extract to suppress vascular development. If lethality is out of context, lower concentrations of the extract must be explored for future studies in order to avoid structural discrepancy of the model organisms. Furthermore, it is suggested that comparative study must be conducted on different sources of Calabash plant so as to examine if the location of the plant plays a key factor in exhibiting extreme toxicity among duck specimens; as referred from Chandra-Sekhar et al., [9].

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