An exploratory study on the anti-angiogenic property of *Ficus benjamina* crude methanolic leaf extract using duck (*Anas platyrhynchos*) chorioallantoic membrane (CAM) assay

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Abstract

Anti-angiogenesis is the inhibition of the growth of blood vessels. *Ficus benjamina* is a tree classified under the Moraceae family, and can be frequently seen in households in the Philippines where it commonly grows and is native to Asian countries. The duck chorioallantoic membrane (CAM) assays were treated with positive control (Doxycycline), negative control (Distilled Water), and crude methanolic leaf extract of *Ficus benjamina* in 3 different concentrations (100 mg/disc, 10 mg/disc, 1 mg/disc). After 72 hours of treatment, the following parameters: total number of branches and total branches length (in pixels), were measured using the ImageJ software. Results show that the values for both parameters of blood vessel growth in CAM assays treated with Treatment 1 (100 mg/disc) were comparable to that of the positive control (Doxycycline). Therefore, the crude methanolic extract of Ficus benjamina exhibits strong anti-angiogenic activity.

Keywords: *anti-angiogenesis, CAM Assay, Ficus benjamina, Moraceae, ImageJ*

Introduction. The process involving new blood vessel sprouting from existing blood vessels is regulated by a physiological complex mechanism known as angiogenesis [1]. It plays a key role in wound healing but is also present in pathophysiological conditions such as cancer and cardiovascular disease [2], which have the top 2 highest rates of mortality in the United States and worldwide in the year 2016, according to the National Center for Health Statistics. Angiogenesis is stimulated mechanically or chemically, with the latter involving several signaling pathways and proteins widely known as growth factors. These cell signaling pathways have become the main interest for drug research and design [3]. However, toxicity issues arise from the use of anti-angiogenic drugs since several of the drugs employed can cause serious side effects, while others may not achieve maximum therapeutic effect [2]. Natural products derived from plant resources have been extensively used in folk medicine for the treatment of cancer, and have been subjects of several studies on anti-angiogenesis [4]. The sap from Ficus plants is widely-known to be toxic, with some species having an LD50 of 5 g/kg body weight [5]. Several studies have shown that the several parts of a certain species *Ficus carica* Linn. such as its bark, leaf extract [6], leaf latex, and fruit latex [7] all exhibit anti-angiogenic property. Other species such as *Ficus deltoidea* [8] also proven to have anti-angiogenic property. In this study, the anti-angiogenic property of methanolic leaf extract of *Ficus benjamina,* will be tested using the Chorioallantoic Membrane (CAM) Assay. Due to the positive results of all Ficus species that have been previously tested and the assumed correlation between toxicity and anti-angiogenic property, it is expected that *Ficus benjamina* will show inhibition of blood vessel growth. The results will determine whether *Ficus benjamina* crude methanolic leaf extract exhibits angiogenic property. This study focused on determining the presence of anti-angiogenic activity of crude methanolic extract of *Ficus benjamina.* The only parameters measured were the number of branches and total branches length in a specific area in the CAM Assay. Additionally, the phytochemical constituents present in the crude methanolic extract were not previously identified and are subject to further investigation in future studies.This research aimed to evaluate the anti-angiogenic activity of crude methanolic extract of *Ficus benjamina* using Chorioallantoic Membrane Assay. It specifically aims to:

(i) Determine the blood vessel growth in terms of number of branches and total branches' length in *Anas platyrhynchos* (duck) embryo CAM Assay after 72 hours of being treated with Positive control (Doxycycline), Negative control (distilled water), and crude methanolic extract of *Ficus benjamina* in 3 different concentrations (100 mg/disc, 10 mg/disc, 1 mg/disc).

(ii) Compare number of branches and total branches' length in *Anas platyrhynchos* (Duck) embryo treated with Positive control (Doxycycline), Negative control (distilled water), and crude methanolic extract of *Ficus benjamina* in 3 different concentrations (100 mg/disc, 10 mg/disc, 1 mg/disc).

(iii) Determine which concentration of *Ficus benjamina* extract is the most effective in inhibiting angiogenesis.

Methods. *Ficus benjamina Leaf Extract.* Mature *Ficus benjamina* leaves were requested from the Research Outreach Station of the Department of Agriculture and verified at the Bureau of Plant Industry, Region 6. After collection, the fresh leaves were washed with distilled water and air-dried at room temperature for three days. The dried material was finely ground into powder using a blender. Powderized leaves weighing 382.72 grams were soaked into 99.5% methanol with 1:5 weight-to-volume ratio. The solution was sealed air-tight and stored in a dark area. After soaking for 24 hours, the solution was filtered using a funnel and Whatmann's no.1 filter paper. The rotary evaporation was performed at the Saint Gabriel College in Kalibo, Aklan. The concentrated extract was dried using a rotary evaporator at 50˚C for four hours.

Duck Eggs. Fertilized *Anas platyrhynchos* (duck) eggs were acquired from a poultry farm in New Buswang, Kalibo, Aklan. Eggs were rejected if cracked, misshapen, soiled, or unusually small or large [9]. The eggs were purchased 5 days after being laid, then further incubated at 37.5˚C with 62.5% relative humidity for 5 days using an automatically-rotating Digital Analytical Incubator (HHD). The arrangement of eggs in the incubator are assigned through complete random sampling.

Chemicals. Methanol was purchased at Patagonian Enterprises in Iloilo City. Doxycycline discs (Liofilchem) were purchased at the Medical Technology - Microbiological Department of Saint Gabriel College, Kalibo, Aklan.

Windowing. On the fifth day of incubation (10th day from being laid), the eggs were candled using a concentrated light source to locate the air sac and identify the prominent Y-shaped blood vessels. Using the narrow edge of the surgical blade, the chorioallantoic membrane (CAM) was separated from the shell by making a hole, perpendicular to the previously identified Y-shaped blood vessel in the center of the egg. A syringe was used to apply a mild suction to the burr hole in order to replace the air sac and drop the CAM away from the shell. A small window above the identified blood vessel was made on the shell using a surgical blade. The pieces of shell were carefully removed with the aid of forceps.

Introduction of Treatments. The following treatments were prepared and dropped onto circle filter paper discs, 0.5 cm in diameter, which were cut from 0.1 cm thick Whatmann filter paper:

Treatment A: Doxycycline (30 ug/disc)
Treatment B: *Ficus benjamina* Extract (100mg/0.2mL/disc)
Treatment C: *Ficus benjamina* Extract (10mg/0.2mL/disc)
Treatment D: *Ficus benjamina* Extract (1mg/0.2mL/disc)
Treatment E: Distilled Water (0.2 mL/disc)

Immediately after windowing the shell, a paper disc with corresponding treatments above was gently placed on top of the previously identified Y-shaped vascular area. The procedure was done inside a laminar-flow hood with UV light. After placing the disc, the window was sealed with sterile transpore or taped-gauze. The eggs were returned to the incubator after all have been treated.

Transferring to Petri dish. Three days after treatment, the eggs were taken out, opened, and transferred to Petri dishes. Then, a plastic ring 1-inch in diameter was placed on the area around the Y-shaped area where the paper disc was planted on. This was done to limit the field of analysis [10].

Image Processing and Software Analysis. A digital camera was used to capture the area of analysis on the CAM [11]. A distance of 6 inches between the camera and the CAM during capture was maintained for all assays. Auto focus was also enabled to ensure clarity of photos. Image processing was done to adjust all pictures to have uniform resolution and number of pixels. This was done at the quality control area of the PBSL-SGC. To eliminate the possibility of human error, the quantification of angiogenesis was made using the "Angiogenesis Analyzer" extension of the ImageJ software [12]. The automatically processed data for number of branches and total length of branches were acquired using this software. ImageJ was downloaded for free online.

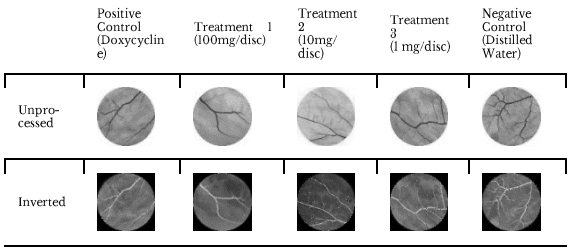
Data Analysis. All calculations were performed using SPSS (version 25; SPSS Inc.). Data was analyzed by one-way analysis of variance (ANOVA), Pearson's Correlation Coefficient, Duncan's New Multiple Range Test (DMRT), and Least Significant Difference (LSD) Test. Significance was accepted at $p<0.01$.

Safety Considerations. For prevention of infection, health damage, and harmful chemicals to contact with skin, protective clothing like laboratory gown, sterile gloves, face mask, and hair net were worn at all times throughout the conduct of the study. To ensure that no contamination occurs, a different syringe was used for each of the treatments.

Ethics. The use of Chorioallantoic Membranes or duck eggs does not require any administrative procedures for acquiring ethics committee approval for animal experimentation [13].

Results and Discussion. *Images Depicting Differences in Blood Vessel Growth.* Selected CAM Assay photos after 72 hours of exposure to different concentrations of the Positive control (Doxycycline), *F. benjamina* Treatment 1 (100 mg/disc), 2 (10 mg/disc), 3 (1 mg/disc), and the Negative control, respectively, are seen in Table 1. Through visual qualitative observation, the blood vessel growth of Treatment 1 and the Positive control are comparable. Additionally, it is evident that the blood vessel growth of Treatment 1 and the Positive control are significantly lower than that of the Negative control.

Table 1. Cropped photographs of circular area enclosed in a plastic ring placed on the CAM Assay.



Mean Blood Vessel Growth. The following are the means of the values generated by the ImageJ software for the two parameters: total number of branches and total length of branches.

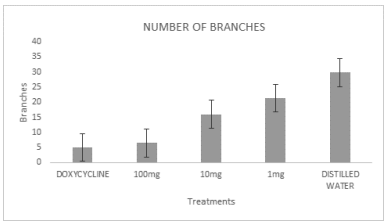


Figure 1. Graph of the mean total number of blood vessel branches of Treatments 1, 2, and 3, Positive and Negative control.

Total Number of Branches. The results showed that Treatment 1 (100 mg/disc) had the mean number of branches of 6.53, Treatment 2 (10 mg/disc) had an average of 16.00 branches while Treatment 3 (1 mg/disc) had 21.33. The Positive control (Doxycycline) had the least number of branches with an average of 5.07 while the Negative control (distilled water) had an average of 29.80 branches, the highest out of all the treatments used.

Table 2. Mean Number of Blood Vessel branches of Treatments 1, 2. and 3, Positive and Negative Controls in terms of number of branches.

Treatment 1 (100mg/disc)	Treatment 2 (10 mg/disc)	Treatment 3 (1 mg/disc)	Positive Control (Doxycycline)	Negative Control (Distilled Water)
6.54 branches	16.00 branches	21.33 branches	5.07 branches	29.80 branches

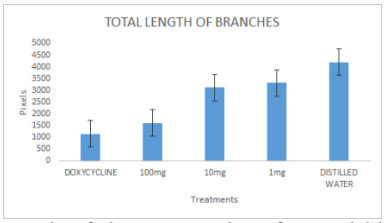


Figure 2. Graph of the mean values for total blood vessel branches' length of Treatments 1, 2, .and 3, Positive and Negative Controls in terms of pixels

Total Length of Branches. Treatment 1 (100 mg/disc) had a mean total branches' length of 1613.06 pixels, Treatment 2 (10 mg/disc) had a mean of 3121.00 pixels while Treatment 3 (1 mg/disc) had the mean of 3316.27 pixels. The positive control of the study had the least mean total branches' length with 1149.73 pixels while the negative control showed a mean amount of 4188.93 pixels and the highest total branches' length out of all the treatments used.

Table 3. Mean Total Blood Vessel branches' length of Treatments 1, 2, and 3, Positive and Negative Controls in terms of pixels

Treatment 1 (100mg/disc)	Treatment 2 (10 mg/disc)	Treatment 3 (1 mg/disc)	Positive Control (Doxycycline)	Negative Control (Distilled Water)
1613.07 pixels	3121.00 pixels	3316.27 pixels	1149.73 pixels	4188.93 pixels

Comparison of Treatments. One-way ANOVA was used to determine whether significant differences in blood vessel growth can be found among the five treatments. Using DMRT, a post-hoc test was done to measure specific differences between pairs of means. Complete tables of statistical analyses can be found in Appendix C.

One-Way ANOVA. One-way ANOVA showed significant difference for both the total number of branches and the total length of branches at p=0.01.

Duncan's Multiple Range test (DMRT). For the total number of branches, the result for Treatment 1 (100mg/disc) and Doxycycline is 0.264 which is higher than the level of significance (p=0.01). For the total length of branches, Treatment 1 (100mg/disc) and Doxycycline is 0.197 which is lower than the level of significance (p=0.01). Meanwhile, Treatment 2 (10mg/disc) and Treatment 3 (1mg/disc) and water is 0.012 which is higher than the level of significance (p=0.01).

Least Significant Difference (LSD). For the total number of branches, the result for Treatment 1 (100mg/disc) and Doxycycline is 0.339 which is higher than the level of significance (p=0.01). For the total length of branches, Treatment 1 (100mg/disc) and Doxycycline is 0.231 which is higher than the level of significance (p=0.01). Meanwhile, Treatment 2 (10mg/disc) and Treatment 3 (1mg/disc) is 0.572 and Treatment 3 (1mg/disc) and water is 0.013 which is higher than the level of significance (p=0.01).

Pearson Correlation Coefficient of different concentrations of F. benjamina leaf extracts. The Pearson Correlation Coefficient result of the three treatments for blood vessel branches count is -0.96514. Meanwhile, for the total branches length, the result was negative 0.99974.

Discussion. In terms of number of both number of branches and total branches' length in duck CAM Assay after 72 hours, the Positive control (Doxycycline) exhibited the lowest mean, followed by Treatment 1 (100 mg/disc), Treatment 2 (10 mg/disc), Treatment 3 (1 mg/disc), and lastly, the Negative control (distilled water). The blood vessel growth of all *Ficus benjamina* extract concentrations are lower compared to the Negative control; therefore, the *Ficus benjamina* extract diluted in distilled water inhibits blood vessel growth more effectively than distilled water alone. The size of the standard deviation error bars reflect the reliability of the mean value as a representative number for the data set. In the error bar graphs shown in Figure 3.1, it can be observed that the error bars for all treatments are long, both for number of branches and total length. This means that all values have a large standard deviation from the mean. However, this does not necessarily indicate that the data are invalid, as biological measurements are usually variable. An overlap in the error bars of two different treatments implies that a significant difference exists between the mean values of the two groups. This is supported by the results of the post-hoc analysis. Post-hoc analysis shows that there is no significant difference between the blood vessel growth of the Positive control (Doxycycline) and Treatment 1 (100mg/disc), both in terms of the total

number of branches and total branches' length. This implies that the anti-angiogenic property of the highest concentration of *Ficus benjamina* extract is comparable to the effectivity of the Positive control. Therefore, the higher the concentration of the *Ficus benjamina* crude methanolic extract, the lower the blood vessel growth, both in terms of number of branches and total branches' length.

Error Analysis. A software was used for the counting of number of blood vessels which could have been a source of gross errors. Systematic errors might have also occurred due to environmental conditions

Conclusion. For both number of branches and total branches' length, the higher the concentration of the *Ficus benjamina* crude methanolic extract, the higher the anti-angiogenic activity of the treatment. Treatment 1 (100 mg/disc) showed a significant difference in results compared to Treatment 3 (1 mg/disc) where Treatment 1 had the lowest number of pixels, out of the 3 concentrations, in terms of number of branches and number of branches' length. Additionally, there is no significant difference in the blood vessel growth of Treatment 1 and the positive control. Therefore, the crude methanolic extract of *Ficus benjamina* exhibits strong anti-angiogenic activity.

Recommendations. To further expand knowledge on the plant species, it is recommended that other types of positive control would be used. The semi-crude extract and the phytochemical constituents of the *Ficus benjamina* leaves should also be analyzed to determine specifically which constituent highly contributes to the anti-angiogenic activity of the *Ficus benjamina* leaf. Lastly, toxicity and lethal dose should be measured and correlated to anti-angiogenic property in future studies.

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