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Der Pharmacia Lettre, 2016, 8 (6):54-58
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

A comparative study on the angiogenic effects of *Artocarpus heterophyllus* Lam. (Langka) and *Artocarpus odoratissimus* Blanco (Marang) crude leaf extracts on chorioallantoic membrane (CAM) of 12-day old duck embryo

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ABSTRACT

The study tested the crude leaf extracts of two plant species under Family Moraceae in 12-day old duck embryo for angiogenic activity using chorioallantoic membrane (CAM) assay. Approximately 300 g of fresh leaves of Artocarpus heterophyllus Lam.(jackfruit) and Artocarpus odoratissimus Blanco(marang) were collected and extracted using 95% ethanol and rotary evaporator. Three concentrations,100ppm, 200ppm, and 300ppm, for each of the leaf extract were prepared and administered on the test eggs. After another 48 hours of incubation, the CAM of test eggs were harvested and collaterals were counted. Results showed that both plant extracts showed anti-angiogenic activity indicated by the decrease in the number of collaterals. In the two plant samples, 300ppm of A. odoratissimus exhibited the highest inhibitory effect; this could be attributed to the presence of phenolic compounds, such as flavonoids, saponins, glycosides, sterols, tannins and anthocyanins.

Key words: *angiogenesis, A. heterophyllus (jackfruit), A. odoratissimus (marang), chorioallantoic membrane (CAM)*

INTRODUCTION

In 2012, there were 8.2 million cancer deaths and 32.6 million living with cancer worldwide [1]. Growth of tumor and metastasis is related to angiogenesis, the formation of new capillaries from pre-existing vessels, since all cancerous tumors release angiogenic growth factor proteins stimulating blood vessels to grow into the tumor providing it with oxygen and nutrients [2] [3]. Therapeutic angiogenesis represents a broad range of interventions that generate new blood vessel growth to promote neovascularization and tissue repair [4]. It is a technique used where blood supply is replenished to provide speed healing. Such technique can save even oxygen-starved hearts [5]. On the other hand, inhibiting angiogenesis could be useful in the treatment of growing tumor or a chronic inflammatory process [6].

Plants had been used for medicinal purposes, including controlling angiogenesis for medical purposes. Several species under family Moraceae, were identified as angiogenesis promoters, such as *Ficus religiosa*[7] [8] or as angiogenesis inhibitors, such as *Morus alba*[9] and *Ficus carica*[10]. The present study determined the effects of different concentrations of jackfruit *(Artocarpus heterophyllus)* and marang *(Artocarpus odoratissimus)* crude leaf extracts on the angiogenesis of 12-day old duck embryos using chorioallantoic membrane assay.

MATERIALS AND METHODS

Collection and Incubation of eggs

Sixty-three 2-day old duck eggs were purchased from a poultry farm located in the municipality of General Trias, province of Cavite. The eggs were placed in an insulated container and transferred at the Biology Research Laboratory of De La Salle University-Dasmariñas for further 10 days incubation. Three duck eggs were used as the test embryos for each treatment with three replicates.

Preparation of Leaf Crude Extracts

Approximately 300 grams of fresh *A. heterophyllus* and *A. odoratissimus* leaves were gathered through hand-picking from the municipality of Silang, province of Cavite. The leaves were washed to be free from dirt and were air dried afterwards. The air dried leaves were then grinded using mortar and pestle, soaked in 70% ethanol for two days before filtration. After filtration, ethanol was evaporated through rotary evaporator at 37°C[11].

The desired concentrations of 100 ppm (T_1), 200 ppm (T_2), and 300 ppm (T_3) of *A. heterophyllus* and *A. odoratissimus* leaves crude extract were prepared using the dilution formula: $C_1V_1=C_2V_2$, where C_1V_1 are the initial concentration and volume of the extract and C_2V_2 are the desired final concentration and volume.The dilution factor was obtained by getting the difference between the final and initial volume[12].

Administration of Leaf Crude Extracts

At the end of the 12-day incubation ~~period~~, a total of 63 viable eggs were selected, 9 eggs for each treatment, to be the test embryos. The selected test eggs were removed from the incubator and were placed in a laminar flow cabinet. Each test egg was swabbed with 70% alcohol and using a 2-ml syringe, a small hole at the pointed end of the shell was made to puncture the air sac. Through the small hole opening on each test egg, 0.3 ml of the different concentrations of crude leaf extract were administered. The opening was resealed by sterilized adhesive tape and the eggs were returned to the incubator horizontally for further two days at 37°C and 70% humidity[13].

Data Gathering and Statistical Analysis

Two days after the administration of *A. heterophyllus* and *A. odoratissimus* leaf crude extract to the developing duck embryo, each of the test eggs was sacrificed and their CAM was prepared for observation of angiogenic activity. The CAM was spread thinly in a petri dish and observed under stereomicroscope (Nikon®C-LED). Collaterals or formed branch points from four randomly selected areas of each CAM were counted and tabulated to compare their angiogenic effects [12].

To determine the significant difference in the angiogenic effects of different concentrations of *A. heterophyllus* and *A. odoratissimus* leaf crude extract on the CAM of duck embryos, one-way analysis of variance was employed. Whenever there is significant difference, Scheffe method was used to compare individual treatment means at 5% probability level.

RESULTS AND DISCUSSION

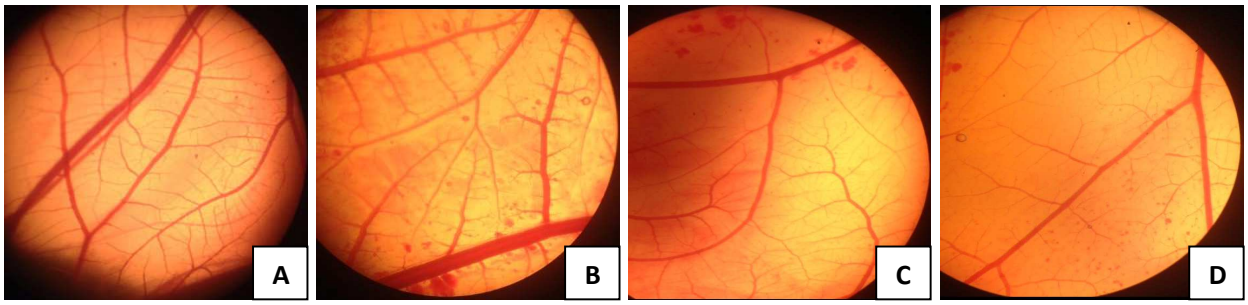
The angiogenic effects, in terms of average number of formed branch points or collaterals, on the CAM of 12-day old duck embryosof three different concentrations of *A. heterophyllus* and *A. odoratissimus* leaf crude extracts are shown in Table 1.

Results showed that there was a decreasing pattern in the number of collaterals of the test eggs with the increasing concentrations of *A. heterophyllus* and *A. odoratissimus* crude leaf extracts. The control group has the highest average collateral number at 103.57 collaterals per egg test followed by those treated with 100ppm with an average of 80.57 and 79.00 collaterals for *A. heterophyllus* and*A. odoratissimus*, respectively. The lowest count of collaterals were those treated with 300 ppm with only an average of 58.85 for those treated with *A. heterophyllus* and only an average of 19.71 for those treated with *A. odoratissimus* leaf crude extract.

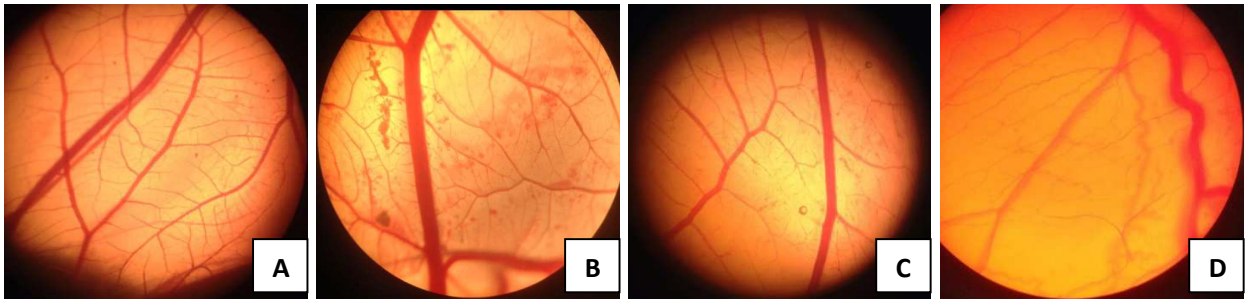
Table 1. Average number of collaterals under the influence of different concentrations of *A. heterophyllus* and *A. odoratissimus*leaf crude extracts

Treatment	*A. heterophyllus*	*A. odoratissimus*
0 ppm(control)	103.5714^{AX}	103.5714^{AX}
100 ppm	80.5714^{BX}	79.0000^{BX}
200 ppm	70.8571^{BX}	51.7143^{CY}
300 ppm	58.8571^{CX}	19.7143^{DY}

Letters ABC and D compared the different concentrations of each leaf crude extract (rows) while letters X and Y compared the two plant samples (columns). Different letters indicate significant difference ($p<0.05$).



Treated with A. heterophyllus leaf crude extract



Treated with A. odoratissimus leaf crude extract

Figure 1. Photomicrographs (45x) of CAM showing formed branch points treated with different concentrations: control (A), 100 ppm (B), 200 ppm (C), 300 ppm (D) of *A. heterophyllus*and *A. odoratissimus*leaf crude extract

Statistically, both leaf crude extracts were able to significantly inhibit the formation of collaterals in comparison with the control group. For those treated with *A. heterophyllus* leaf crude extract, 100 ppm and 200 ppm concentrations induced the same inhibitory effects in the formation of collaterals. While for those treated with *A. odoratissimus*, all experimental treatments exhibit significant difference in the number of collaterals, i.e. decreasing number of branch points as the concentration increases. The 300 ppm concentration for both leaf crude extract has the greatest inhibitory angiogenic effects by having the lowest number of formed branch points as compared with the other experimental treatments.

These results showing inhibitory angiogenic effects of *A. heterophyllus* could be attributed to the phenolic compounds, such as flavonoids, saponins, glycosides, sterols, tannins and anthocyanins, present in its leaf crude extract [12]. Flavonoids are said to be angiogenic inhibitors. It inhibits vascularization process since they are able to interfere in the steps of angiogenesis such as destruction of blood vessels, proliferation as well as migration making it suitable also for blocking tumor growth [15]. This is also similar to the findings about the anti-tumor activity of crude extracts from tegmen that exhibited cytotoxicity [16]. Saponins are also known to have an anti-angiogenic effect. Its effect was tested on different assays like CAM assay, rat aorta ring assay, and human umbilical vein endothelial cells assay and showed significant anti-angiogenic effect [17]. Glycosides have also the potential in anti-angiogenic and anti-tumor activity. Glycosides were active in vitro angiogenic assays by inhibiting the endothelial

cells' invasion and migration [18]. Sterols act through many mechanisms of action like inhibition of carcinogen production, inhibition of growth of cancer cells, apoptosis of cancer cells, invasion, and especially, inhibition of angiogenesis [19]. Phenolic compounds are proven to have an anti-angiogenic property. Tannins inhibit the matrix enzymes which are responsible for malignant tumor growth and invasion [20].

On the other hand, *A. odoratissimus* also contains phenolic compounds such as flavonoids, arylbenzofurons, stilbenoids, and lectin called jacalin, which are richly found in *Artocarpus* species. These metabolites have many important bioactive compounds that aid in biological activities like inflammation and cytotoxicity making them effective angiogenic inhibitors [21].

Comparison between the two plant samples reveal that at 100ppm, *A. heterophyllus* and *A. odoratissimus* showed no significant difference in their abilityto inhibit angiogenesis. However, for those treatments with higher concentrations, 200 ppm and 300 ppm, *A. odoratissimus* significantly inhibit angiogenesis with lower number of collaterals as compared to those treated with *A. heterophyllus*. These results could be due to the high levels of potent phytochemicals that are present only *A. odoratissimus*, like the compound artosimmin[22].

CONCLUSION

Both *A. heterophyllus* and *A. odoratissimus* showed anti-angiogenic or inhibitory effect on the vascularization on chorioallantoic membrane (CAM) of 12-day old duck embryos. As the concentrations of the crude leaf extracts were increased, lower number of collaterals were formed; those treated with 300 ppm of both leaf extracts had the lowest number of collaterals formed. However, at 100ppm, the two *Artocarpus* speciesshowed no significant difference in their ability to inhibit angiogenesis. Only at 200 ppm and 300 ppm, *A. odoratissimus* significantly inhibit angiogenesis with lower number of collaterals as compared to those treated with *A. heterophyllus* at the same concentrations. These findings are attributed to the presence of phenolic compounds, such as flavonoids, saponins, glycosides, sterols, tannins and anthocyanins in the leaves of both species.

Acknowledgments
The suggestions and helpful critique of A/Prof Cherry Cuevas, A/Prof Janette Bala and Dr. Jonathan Rubio of the Biological Sciences Department, De La Salle University-Dasmariñas are duly acknowledged.

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