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Screening for the anti-angiogenic activity of selected Philippine medicinal plants using chorioallantoic membrane assay

*J.E. Camposano, G.L.T. Dela Torre, J.G. Laxamana, L.L.H. Larcia II**
College of Pharmacy, University of the Philippines – Manila, Ermita, Manila, 1000, Philippines

Abstract

The search for possible cure against angiogenesis-dependent diseases is focused on plants because drugs derived naturally are inherently better tolerated in the body than synthetic drugs. *Quisqualis indica*, *Carmona retusa* and *Peperomia pellucida* underwent *ex ovo* chorioallantoic membrane (CAM) assay to determine their anti-angiogenic potential, which can provide new avenues in treating angiogenesis-dependent diseases. The crude methanolic extracts of the three plants exhibited inhibition of angiogenesis with the highest activity observed on *C. retusa* (49.92 $\pm$ 1.53%), followed by *Q. indica* (29.78 $\pm$ 5.93%) and *P. pellucida* (26.81 $\pm$ 1.56%). The *C. retusa* crude methanolic extract was subjected to phytochemical analysis and was revealed to contain alkaloids, carbohydrates, phenols, tannins and sterols. After performing one-way ANOVA and Tukey's post-hoc test (significant at $p < 0.05$), it was noted that the *C. retusa* crude methanolic extract has a comparable activity with the positive control, Quercetin (60.24 $\pm$ 3.43%). The results suggest that *C. retusa* can be a potential source of anti-angiogenic compound for the design and development of drugs targeting angiogenesis-dependent diseases.

Keyword: *Carmona retusa; Ex ovo* chorioallantoic membrane assay; Anti-angiogenic activity; Quercetin

1. INTRODUCTION

Angiogenesis is the biological process by which new blood vessels are formed from pre-existing capillaries and post-capillary venules[1]. The formation of new blood vessels needs to be highly regulated because blood vessels that grow too exuberantly can have detrimental effects. Diseases that are related to excessive angiogenesis include retinopathy, liver cirrhosis, psoriasis and cancer[2]. Since the process plays an important role in these pathological conditions, inhibition of angiogenesis is one promising approach in their therapy and management. Synthetic angiogenesis inhibitors are available in the market, however, they are costly and there have been records of resistance to anti-angiogenic therapy[3]. Since phytomedicines have fewer side effects, there is an increased interest in discovering natural compounds that exhibit anti-angiogenic activity to address the complications brought by synthetic drugs[4].

The Philippine Department of Health (DOH) approved ten herbal medicines recommended for human use as home remedies for different diseases. Three of these herbal plants have not been studied for their anti-angiogenic potential namely, *Quisqualis indica* (Niog-niogan), *Carmona retusa* (Tsaang Gubat) and *Peperomia pellucida* (Ulasimang Bato). Their uses were described by the Philippine Institute of Traditional and Alternative Health Care (PITAHC)[5]. *Q. indica* is traditionally used as an anthelmintic. *C. retusa* is approved by the DOH as stomachic. *P. pellucida* on the other hand is used to reduce uric acid in the blood especially for the prevention and treatment of gouty arthritis. With the established uses of the three aforementioned plants, there is still a need to discover their other potential medicinal uses to optimize their therapeutic capacity and

***Corresponding author:** lhlarcia@up.edu.ph

the lack of studies about these herbal plants as angiogenesis inhibitors provide opportunities in the discovery of drugs under this class. The three plants were chosen in the present study because they have good antioxidant activity[6-8]. Antioxidant decrease the generation of reactive oxygen species (ROS) in the biological system. These ROS play a critical role in the pathologies of angiogenesis-dependent diseases as they initiate the formation and growth of blood vessels[9]. Antioxidants, thus in part, contribute to the inhibition of angiogenesis. A strong link between potent antioxidants and anti-angiogenesis were identified by a number of studies suggesting that natural product compounds and extracts with promising antioxidant activity might show anti-angiogenic capacity[10-13].

The anti-angiogenesis research utilizes the chorioallantoic membrane (CAM) of the avian embryo. The CAM model has many advantages including its low cost, ease of use, high reproducibility, reliability and simplicity[14]. The CAM assay, in comparison with other animal models, is a closed system, which promotes a longer half-life on many compounds because excretion of such compounds is eliminated. This, in turn, allows the use of minute amounts of experimental compounds[15]. It also offers a quasi-two-dimensional structure of the vasculature tree and transparency that is well-suited for imaging the inhibition of blood vessel growth[16]. Quantification of blood vessels in large amount of CAM models can be used to screen drugs from sample plant extracts[17]. In CAM assay, quercetin can be used as the positive control. Quercetin is a dietary flavonoid that has the capacity to suppress vasculature growth *in vitro* and *in vivo* by affecting the steps of the angiogenesis process[18]. Blood vessels become dense around the applied anti-angiogenic test compound or even disappear.

The potential uses of the three aforesaid herbal plants need to be explored. Their anti-angiogenic activity through the CAM assay was studied to provide new opportunities and avenues in the field of therapy for angiogenesis-dependent diseases, especially cancer therapy which can address the morbidity and mortality they cause.

2. MATERIALS AND METHODS

2.1. Plant and Animal Samples

2.1.1. Collection of Plant Samples and Extraction

Fresh mature leaves of *Q. indica* were collected from Raymundo Gate, College, Los Baños, Laguna; fresh mature leaves of *C. retusa* were obtained from the Department of Agronomy, University of the Philippines, Los Baños, Laguna; and the aerial parts of *P. pellucida* were collected from Barangay 2, Alabat, Quezon. Authentication of the plant samples was performed by the Bureau of Plant Industry, Philippines.

Fresh mature leaves of *Q. indica* and *C. retusa* and fresh aerial parts of *P. pellucida* were garbled, washed, air-dried and cut into small pieces. Samples were macerated separately for seven days using methanol as the extracting solvent. After seven days, the methanol soluble materials were filtered off. The filtrates were concentrated *in vacuo* at a maximum temperature of 40°C using a rotary evaporator. The concentrated filtrates were transferred in evaporating dishes and were evaporated to dryness in a water bath.

2.1.2. Preparation of Test Samples

The crude methanolic extracts were dissolved in distilled water to yield a solution with a concentration of 10 mg/mL. For the preparation of discs, 10 µL of these solutions was applied dropwise using a 10 µL capillary pipette on a sterile filter paper of 3 mm diameter, dried under a laminar flow hood to achieve a concentration of 100 µg/disc. Quercetin, at a concentration of 100 µg/disc was used as the positive control, while 10 µL of distilled water impregnated in the filter paper disc was used as the negative control[14].

2.1.3. Test Animals

Three-day old fertilized duck eggs were collected from Burol, Villa Juliana, Balagtas, Bulacan. Dirt, feathers and excrements were carefully removed from the exterior of the eggs using distilled water. Eggs weighing at least 50 g were chosen. Egg candling was performed in order to determine

if the eggs were fertilized. An egg is confirmed fertilized if a tiny speck on the yolk called the germ spot is present during egg candling. Fertilized eggs were then incubated for a day for acclimatization at a relative humidity of 60-62% and temperature of 37°C. The eggs were used for the Chorioallantoic Membrane (CAM) Assay[19]. The test animals were handled in accordance with good animal practice as approved by the Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines, Manila.

2.2. Chorioallantoic Membrane (CAM) Assay

The test specimens were divided into five treatment groups: one group for the negative control, one group for the positive control and three groups for the crude methanolic extracts. Each group consists of ten sample-infused filter paper discs to be applied to the duck's CAM.

After a day of acclimatization, the eggs were wiped individually with 70% ethanol and were transferred to a biosafety cabinet. The eggs were horizontally held and were cracked on the edge of a previously autoclaved steel spatula oriented perpendicularly to the eggs. The contents of each egg were transferred to a sterile petri dish by applying pressure through the crack and carefully separating both halves of the eggshell. The *ex ovo* cultures were returned to the incubator with minimal disturbances and dead embryos were immediately removed[20]. These steps were performed in order to minimize infection on the CAM, increasing their survival rates.

The number of blood vessels of the five-day old embryos was counted prior to the application of the filter paper discs. The discs were placed directly using a sterile forceps over the blood vessels on the growing CAM. The *ex ovo* cultures were returned to the incubator. The final evaluations were carried out the next day[19].

The filter paper discs were gently removed from the six-day old embryos and the number of blood vessels was determined at the site of sample application. To ease the data collection procedure, the number of blood vessels determined from the photographs of the cultures before and after treatment was compared. The percent inhibition for each test samples was computed using the formula:

$$\text{Percent Inhibition} = \frac{X - Y}{X} \times 100$$

wherein X is the number of blood vessels of the untreated CAM and Y is the number of blood vessels of the treated CAM[21].

2.3. Phytochemical Analysis

The crude methanolic extract of the plant that exhibited the highest anti-angiogenic activity was subjected to phytochemical analysis[22-24].

2.3.1 Test for Alkaloids

The extract was treated with 3 to 5 drops of different alkaloidal reagents (Wagner's, Valser's, Mayer's, Hager's and Dragendorff's reagent). Presence of alkaloid is indicated by the precipitation, which is reddish-brown in Wagner's, white in Valser's and Mayer's and prominent yellow in Hager's and Dragendorff's reagent.

2.3.2 Test for Carbohydrates

Few drops of Molisch's reagent were added to 2 mL of the extract, followed by the addition of concentrated sulfuric acid down the side of the test tube. Formation of a red or dull violet color at the interphase indicated a positive result.

2.3.3 Test for Reducing Sugars

Five mL of diluted sulfuric acid was added to the extract and boiled for fifteen minutes in a water bath. It was cooled and neutralized with 20% potassium hydroxide solution. A mixture of 10 mL of equal parts of Fehling's A and B were added and then boiled for five minutes. A dense red precipitate indicates the presence of reducing sugar.

2.3.4 Test for Flavonoids

Two mL of the extract was treated with few drops of 20% sodium hydroxide

solution. Formation of intense yellow color, which becomes colorless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

2.3.5 Test for Phenols

A fraction of the extract was treated with 5% aqueous ferric chloride. Formation of deep blue or black color indicated the presence of phenols.

2.3.6 Test for Tannins

Two mL of the extract was treated with 10% alcoholic ferric chloride solution. Formation of blue or greenish color indicated the presence of tannins.

2.3.7 Test for Saponins

To 2 mL of the extract, 6 mL of water was added in a test tube. The mixture was vigorously shaken and was observed for the formation of persistent foam that confirms the presence of saponins.

2.3.8 Test for Sterols

To 1 mL of the extract, few drops of chloroform, acetic anhydride, and concentrated sulfuric acid were added. Formation of a dark pink or red color in the solution indicated the presence of sterols.

2.3.9 Test for Terpenoids

One mL of chloroform was added to 2 mL of the extract followed by a few drops of concentrated sulfuric acid. A reddish brown precipitate produced immediately indicated the presence of terpenoids.

2.3.10 Test for Quinones

A small amount of the extract was treated with concentrated hydrochloric acid. Formation of yellow precipitate indicated the presence of quinones.

2.3.11 Test for Oxalates

To 3 mL portion of the extract, few drops of glacial acetic acid were added. A greenish black coloration indicated the presence of oxalates.

2.4 Statistical Analysis

Ten filter papers per test samples and controls were used in the experiment to obtain data for the percentage inhibition which were expressed as Mean $\pm$ S.E.M. The differences between groups were compared by one-way ANOVA followed by Tukey's post-hoc test and considered significant at $p < 0.05$. The statistical analysis was carried out using SPSS edition 17.0.

3. RESULTS

3.1 CAM Assay of the Crude Methanolic Plant Extracts

The anti-angiogenicity of the crude methanolic extract of *Q. indica*, *C. retusa* and *P. pellucida* were tested through *ex ovo* CAM model. Counting of blood vessels were conducted before and after the treatment within the area covered by the filter paper discs. Summary of the results is shown in Table 1 and Figure 1. On macroscopic examination of the CAMs treated with distilled water (Figure 2), normal vascular architecture was observed with prominently branched-out, well-developed blood vessels. An inconspicuously minimal inhibition of 2.08 $\pm$ 4.03% was observed on the blood vessels of CAMs treated with distilled water. Quercetin, a known anti-angiogenic compound, was used as the positive control. In the quercetin treated CAMs (Figure 3), gross changes in the vascular architecture were observed. There was a decrease in and inhibition of the blood vessel branching which was quantitatively determined to be 60.24 $\pm$ 3.43%. For the methanolic extracts, inhibition of blood vessel formation was observed at a concentration of 100 µg. After 24 hours of treatment, branching pattern of the blood vessels below the disc containing the extracts were observed to disappear. *Q. indica* (Figure 4A) and *P. pellucida* (Figure 4C) displayed an inhibition of 29.78% $\pm$ 5.93% ($p < 0.05$) and 26.81 $\pm$ 1.56% ($p < 0.05$), respectively. The highest inhibition was

observed in the *C. retusa* treated group (Figure 4B), with an inhibition of 49.92% ± 1.53% ($p > 0.05$), comparable to the activity of the positive control, quercetin. The crude methanolic extract of *C. retusa* was then subjected to phytochemical analysis.

Table 1. Percentage inhibition of blood vessel formation by the three crude methanolic extracts (ME) together with the positive control, quercetin and negative control, distilled water.

Treatment Groups	Percentage Inhibition (Mean + S.E.M.)
Quercetin	60.24 + 3.43
Distilled Water	2.08 + 4.03
Quisqualis indica ME	29.78 + 5.93
Carmona retusa ME	49.92 + 1.53
Peperomia pellucida ME	26.81 + 1.56

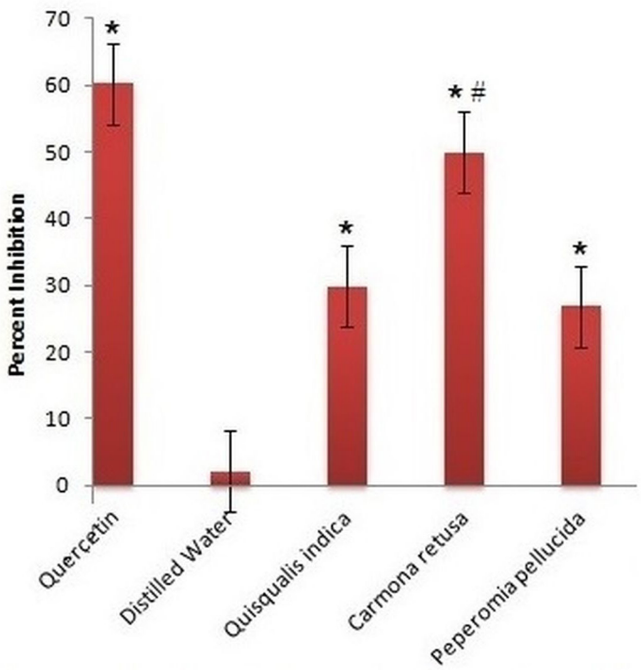

Figure 1. Parcentage inhibition of blood vessel formation od the crude methanolic extracts and controls.
* significantly different ($p < 0.05$) with Distilled Water.
# insignificantly different ($p < 0.05$) with Quercetin.

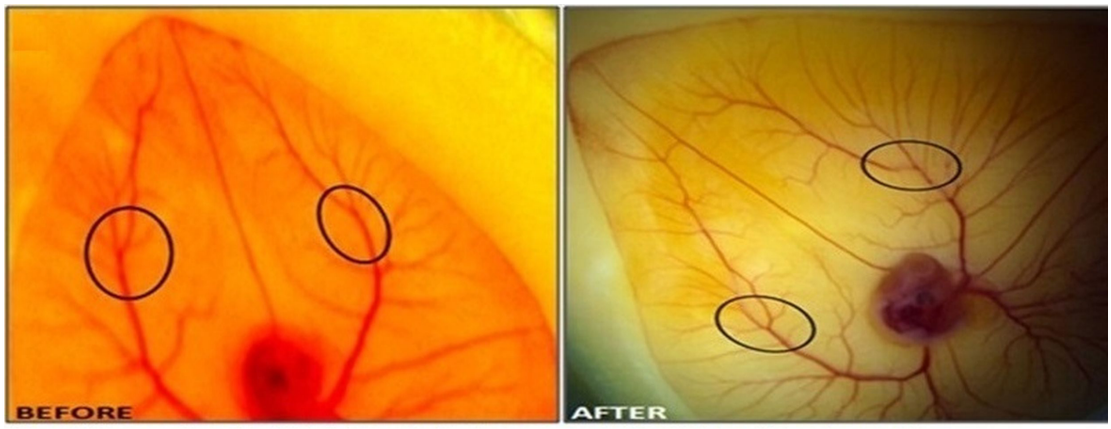

Figure 2. Photographs of the CAM before and after the treatment of Distilled Water (Negative control).

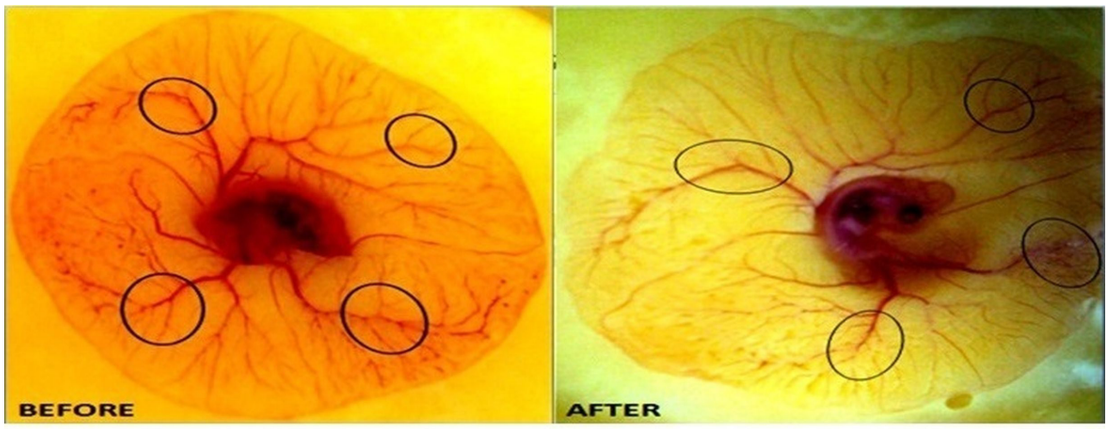

Figure 3. Photographs of the CAM before and after the treatment of Quercetin (Positive control).

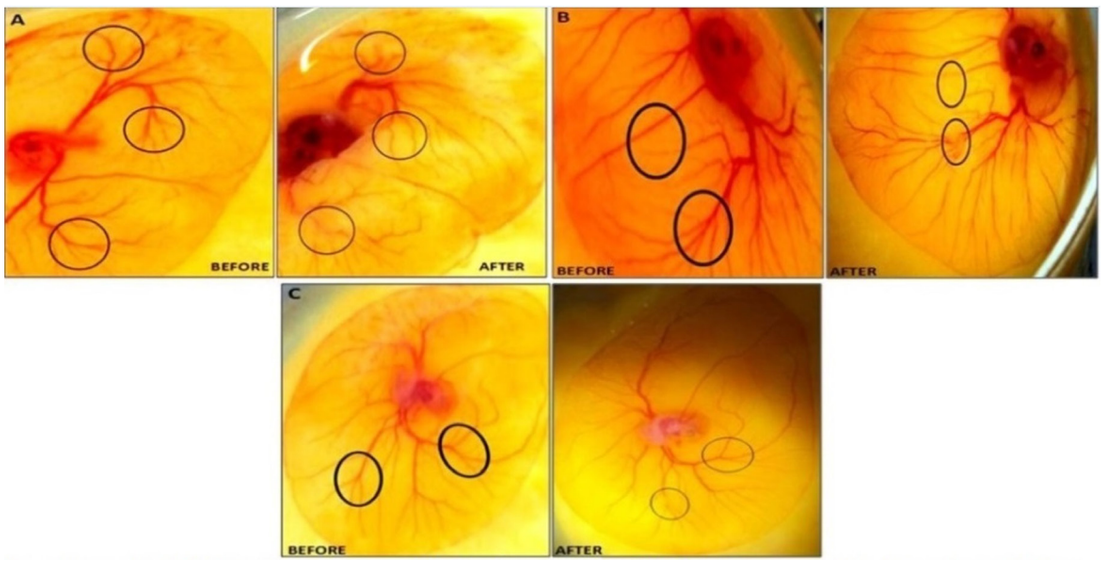

Figure 4. Photographs of CAMs before and after the treatment of the methanolic extracts of *Quisqualis indica* (A), *Carmonaretusa* (B) *and Peperomia pellucida* (C).

Table 2. Results of the phytochemical analysis of *Carmona retusa* methanolic extract

Phytochemicals	Result
Alkaloids	+
Carbohydrates	+
Reducing Sugars	-
Tannins	+
Flavonoids	-
Phenols	+
Saponins	-
Sterols	+
Terpenoids	-
Quinones	-

* (+) presence; (-) absence

3.2. Phytochemical Analysis

A qualitative analysis was employed for the screening of several phytochemicals (Table 2). Analysis of the crude methanolic extract of *C. retusa* revealed the presence of alkaloids as shown by the positive results observed with Wagner's test, Hager's test and Dragendorff's test. The presence of carbohydrates was confirmed using Molisch reagent, while the occurrence of phenols and tannins were shown by the positive results with ferric chloride. Lastly, the presence of sterols was indicated by a positive result in Liebermann-Burchard test.

4. DISCUSSION

Persistent upregulated angiogenesis is one of the hallmarks of cancer and a common feature in diseases like diabetic retinopathy, atherosclerosis and rheumatoid arthritis[25]. Therefore, interruption and blockage of this process is an avenue in the management of the aforementioned angiogenesis-dependent diseases. Our present study, focuses on the search for sources of natural-based anti-angiogenic compounds.

Ex ovo chorioallantoic membrane assay is a method of accessing the anti-angiogenic potential of a compound in a CAM with minimal invasion. The embryo is cultured in a petri dish, which provides a better access to the test site, thus improving the ability to repetitively treat or to have a multiple test sites on one CAM[26]. Three-day old duck eggs were used in the assay since in the eggs at later developmental stages (> 72 hours), the yolk sacs are thicker which tends to adhere to the egg shell which may lead to small hemorrhages at the area of adherence or the rupture of the yolk sac membrane[20]. This can lead to the difficulty in transferring the CAM in petri dishes and lower their survival rates.

In the present study, CAMs treated with the methanolic extracts of *Q. indica, P. pellucida* and *C. retusa* elicited inhibition of blood vessel formation, with the highest activity observed in *C. retusa*. Since the highest inhibition was observed in *C. retusa*, it was subjected to phytochemical analysis. Alkaloids, carbohydrates, phenols, tannins, and sterols were identified to be present. Alkaloids are found to exhibit strong anti-angiogenic activity, and these alkaloids may act through different mechanisms to inhibit angiogenesis[25,27]. Phenolic compounds are known antioxidant compounds which were found to elicit their anti-angiogenic activity by preventing the ROS to initiate and progress the angiogenesis cascade[28,29]. Similarly, some naturally occurring tannins, carbohydrate derivatives and sterols

also demonstrate activity through impeding the angiogenesis system[30-32]. These suggest that the presence of these secondary metabolites in *C. retusa* methanolic extract indicates the potential of the medicinal plant as a source of anti-angiogenic medicines. The results show significance in the management of angiogenesis-dependent diseases. Possible mechanisms by which the crude methanolic extract of *C. retusa* elicit their activity may be explained by the interferences in the multi-step angiogenesis process such as the induction of apoptosis[9], inhibition in the proliferation and migration of endothelial cells[33] as well as its interaction between different enzymes and growth factors[34]. To identify the exact mechanism of angiogenesis inhibition, further analysis should be conducted using angiogenesis array kits, enzyme-linked immunosorbent assay (ELISA) and *in vivo* matrigel plug assay with histological examination on rats[34].

5. CONCLUSION

Our study suggests a possible source of anti-angiogenic compound which is the *C. retusa* crude methanolic extract. It has a comparable activity with a known angiogenesis inhibitor, Quercetin. Our data demonstrate that alkaloids, carbohydrates, phenols, tannins and sterols are all likely contributors to the observed anti-angiogenic property of the herbal plant and may act in a complementary fashion to inhibit blood vessel formation. Nonetheless, *in vivo* anti-angiogenic assays are necessary in order to support the current findings.

6. ACKNOWLEDGEMENT

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