**Malaysian Applied Biology (2024) 53(2): 72-80**
https://doi.org/10.55230/mabjournal.v53i2.2769

***Research***

# Phytochemical Evaluation and Anti-angiogenic Activity of Alingatong (*Dendrocnide meyeniana* Walp.) Root Extracts Using the Chorioallantoic Membrane Assay on Duck Embryo

**Ezra Maurel D. Torno[1,2], Merrah Joy B. Subebe[2], Muhmin Michael E. Manting[1], Mylah Villacorte-Tabelin[1,2]***

1. Department of Biological Sciences, CSM, MSU-Iligan Institute of Technology, Iligan City, 9200 Lanao del Norte, Philippines
2. PRISM, MSU-Iligan Institute of Technology, Iligan City, 9200 Lanao del Norte, Philippines
*Corresponding author: mylah.tabelin@g.msuiit.edu.ph

**ABSTRACT**

*Dendrocnide meyeniana* (Walp.), known as "Alingatong" in the Visayas region of the Philippines, is a plant from the Urticaceae family. It is commonly found in the mountain areas of Taiwan and the Philippines. This study aims to determine the *Dendrocnide meyeniana* root extract's anti-angiogenic activity. The methanolic extracts of *Dendrocnide meyeniana* were subjected to preliminary phytochemical screening. Cytotoxicity test using Brine Shrimp Lethality Assay was conducted with different plant concentrations to determine the concentration to use for the anti-angiogenic activity of the root extracts. The anti-angiogenic activity was observed by calculating blood vessel percent inhibition using chorioallantoic membrane assay or CAM assay. Phytochemical screening reveals that the plant contains varying levels of detection of bioactive compounds, including saponins, flavonoids, alkaloids, and steroids. Brine Shrimp Lethality Assay showed that the ethyl acetate and hexane extracts from *Dendrocnide meyeniana* were highly toxic as their $LC_{50}$ values were lower than 100 ppm. CAM assay results showed that hexane with 250 ppm concentration has the highest percent inhibition with 34.98% followed by hexane at 125 ppm with 34.07% inhibition. Both concentrations of ethyl acetate showed low percentage inhibition, where 250 ppm was at 19.05% and 125 ppm at 15.93%. One-way ANOVA indicates that the different treatments have significant differences (*p*-value< 0.0001) in the number of branches present using the CAM assay. Therefore, based on the results, *Dendrocnide meyeniana* root extracts have anti-angiogenic properties and these findings will help to understand the efficacy of the traditional medicine used by the local people.

**Key words:** Alingatong, bioactive compounds, cytotoxic, *Dendrocnide meyeniana*, $LC_{50}$, medicinal plants

***Article History***
Accepted: 21 May 2024
First version online: 30 June 2024

***Cite This Article:***
Torno, E.M.D., Subebe, M.J.B., Manting, M.M.E. & Villacorte-Tabelin, M. 2024. Phytochemical evaluation and anti-angiogenic activity of *Dendrocnide meyeniana* Walp. (Alingatong) root extracts using the chorioallantoic membrane assay on duck embryo . Malaysian Applied Biology, 53(2): 72-80. https://doi.org/10.55230/mabjournal.v53i2.2769



## INTRODUCTION

Plants are utilized for medical purposes due to the substances they contain, which can be used to cure or alleviate specific conditions (Salmerón-Manzano *et al*., 2020). Worldwide, many people rely on medicinal plants for primary health care, income generation, and improved quality of life, as they have long served as a source of drugs for treating human illnesses (Belayneh *et al*., 2019). Medicinal plants are widely spread throughout the Philippines, and various distinct local groups hold knowledge of their utilization. Medicinal plants demonstrate a wide range of plant species with therapeutic value, highlighting the crucial role traditional knowledge and practices play in supporting the local healthcare system. Importantly, the reported medicinal uses could be scientifically tested for safety and efficacy, potentially leading to pharmaceutical applications in the future (Caunca & Balinado, 2021). In medicinal plants, phytochemicals such as alkaloids, tannins, and other secondary metabolites, are abundant (Ali *et al*., 2021). One possible application of bioactive compounds is for the treatment of various forms of cancer through inhibition of angiogenesis. Angiogenesis is essential in the human body, from early life to the development of life-threatening diseases such as cancer, heart attack, and

stroke (Kargozar *et al*., 2020). Although angiogenesis is associated with many developmental processes, recent studies have shown that excessive, insufficient, and abnormal angiogenesis contributes to many more pathogenesis disorders (references). Developing new blood vessels can influence many immune, ischemic, inflammatory, infectious, and malignant diseases when dysregulated (Carmeliet, 2000). Bioactive compounds from plants are often used as inhibitors of angiogenesis that can inhibit and interrupt blood vessel formation (Lu *et al*., 2016). These secondary metabolites demonstrate anticancer effects either on their own or in collaboration with other compounds by controlling metabolic and signaling pathways. They inhibit crucial enzymes involved in cancer advancement, angiogenesis microtubule assembly and trigger apoptosis (Ramakrishna *et al*., 2021)

*Dendrocnide meyeniana* is distributed in Southeast Asia, reaching three to five meters in height. It belongs to the Nettle family, recognized for its stinging hairs on the leaves. This plant is traditionally used for treating ailments (Stuart, 2021; Gunardi *et al*., 2023). However, the anti-cancer activity from *Dendrocnide meyeniana*'s roots has not been fully explored. Thus, this study aims to determine whether *D. meyeniana* root extracts have anti-angiogenic activity using the CAM assay. The duck CAM is a quick, low-cost, reproducible, and effective short-term *in ovo* animal model for preclinical cancer research (Lokman *et al*., 2020).

## MATERIALS AND METHODS

### Plant collection and authentication

All plant samples were collected within the parameters of the gratuitous permit (GP No. 2022-15), issued by the Region VII Department of Environment and Natural Resources (DENR) Office. To ensure the identity of the plant species used for herbal medicine by the locals, a representative from the DENR - Community Environment and Natural Resources Office (DENR-CENRO) and local farmers (Latap farmers association) in Zamboanguita, Negros Oriental assisted in the collection. Documentation was done in the form of detailed field notes and photographs that recorded the habit, inflorescence, infructescence, leaf and stem morphology. The plant specimen was given initial identification upon collection and a final verified identification by a botanist from MSU-Iligan Institute of Technology. A voucher specimen was deposited at the University of Santo Tomas Herbarium (USTH-018471).

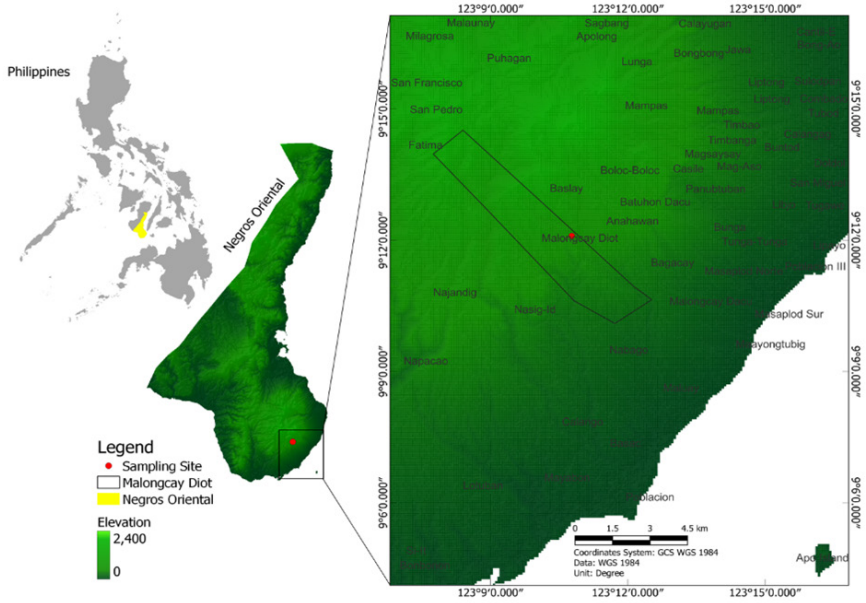


**Fig. 1.** Sampling site at Barangay Malongcay-Diot, Zamboanguita, Negros Oriental Philippines generated QGIS

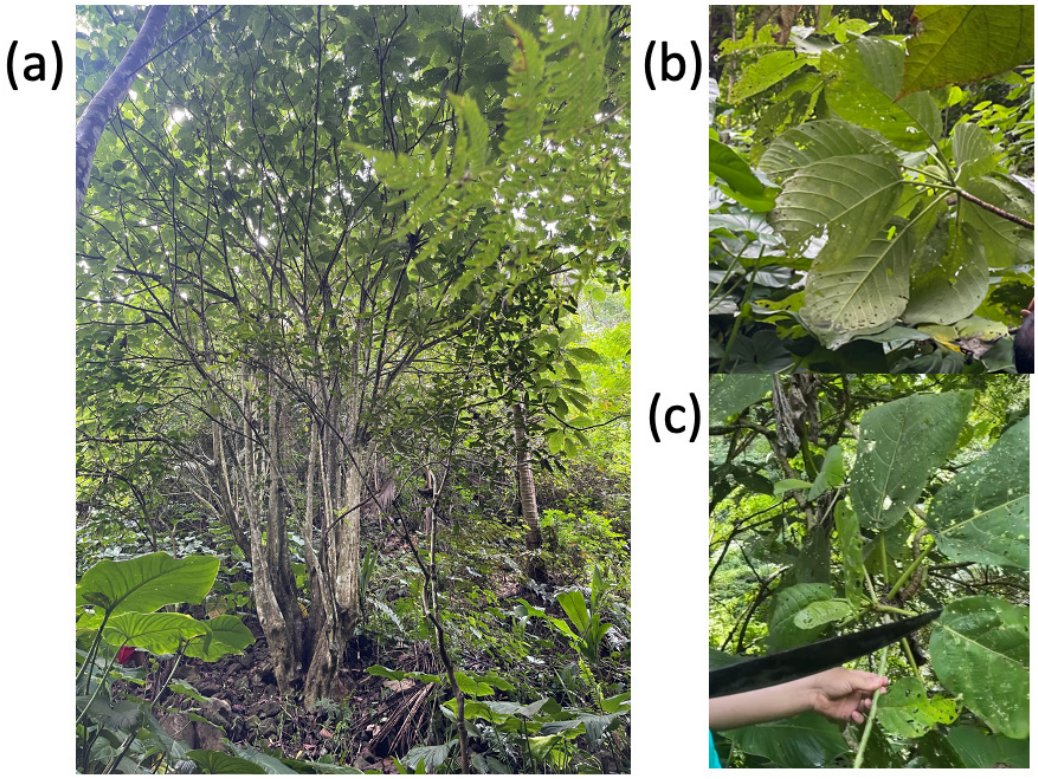


**Fig. 2.** *Dendrocnide meyeniana* plant (a) Upperside of the leaf (b). Lower side of the leaf (c).

## Preparation of plant extracts

*Methanolic crude extract and decoction*

The roots of *D. meyeniana* were air-dried at room temperature for two weeks. The dried root was ground into small particles until powdered using a commercial blender and sieved using a steel mesh sieve. The dry powdered plant root was soaked with absolute methanol with a ratio of 1 g: 5 mL. The soaking was done for 72 hr in a dark place and was filtered using Whatman no. 1 filter paper. Then, the methanolic extract was subjected to a rotary evaporator at 35 °C and freeze-dried.

For the queous solution, a decoction was performed with a 1:2 ratio. Plant roots were boiled for 20 min, filtered with Whatman no 1 filter paper, and subjected to lyophilization or freeze-dried.

For the solvent partitioning, the mixture's chemicals are divided into two categories in this way. In a separating funnel, 100 mL of aqueous extract and 100 mL of hexane were added. The aqueous methanolic at the bottom of the funnel was collected and repeated three times. The aqueous extract was combined with ethyl acetate until partition was already apparent and was repeated three times. At the bottom of the funnel, the aqueous was collected. After that, rotary evaporation and freeze-drying were used to concentrate the fraction.

A stock solution was prepared with approximately 40 mg of each plant extract (methanolic, hexane, ethyl acetate, and aqueous). A 1 mg/mL stock solution was prepared for each extract by dissolving the 40 mg of the plant extract in 40 mL of distilled water. Serial dilution was prepared from the stock solution.

## Phytochemical screening

The phytochemical screening was performed by a Registered Chemist from the Department of Chemistry of the Mindanao State University-Iligan Institute of Technology using a standard protocol with modifications (Claustra *et al.,* 2005).

*a. Test for alkaloid*

2 mg of the crude extract from the prepared stock solution was added to 2 mL of 2 M of HCl. The filtrates were then treated with 1 mL of potassium bismuth iodide solution (Dragendorff's reagent). The formation of an orange precipitate determined the presence of an alkaloid.

*b) Test for flavonoids*

A few drops of 1.0 M sodium hydroxide solution were added to 2 mg of the crude extract. The appearance of a strong yellow color that turns colorless upon the addition of HCl indicates the presence of flavonoids.

*c) Test for saponins*

About 5 mg of plant extract was mixed with 5 mL of distilled water. The solution was shaken vigorously. Persistent frothing indicates the presence of saponin in the extract.

*d) Test for steroids*

2 mg of the plant extract was dissolved with 2 mL of chloroform. An equal volume of concentrated sulfuric acid was added from the side of the test tube. The presence of steroids was detected by the formation of a violet-to-blue-colored ring at the confluence of the two liquids.

*e) Test for tannins*

About 0.5 g of crude extract was mixed in 10 mL of distilled water. The solution was filtered, and 2 mL of 5% ferric chloride was added to the filtrate. The discoloration of the solution to brownish-green or blue-black color indicates the presence of tannins.

The indication is, with a plus (+) sign denoting the existence of a component of the active substance, wherein single (+), double (++), triple plus (+++) and a negative or minus sign (-) for no detection denote varying degrees of detection.

**Brine shrimp (*Artemia salina* L.) lethality assay**

To assess the toxicity of the different plant concentrations, the brine shrimp lethality test (BSLT) was used to determine which solvent and concentration were to be used in the CAM assay.

An artificial seawater was prepared and placed in a small container that serves as the hatching chamber. Shrimp eggs were placed in the hatchery and aerated. After forty-eight hours, the shrimp larvae were collected and put in a different container for preparation for the experiment.

Four (4) mL of the artificial seawater and one (1) mL of plant extract solution were added to each container, and ten brine shrimps were introduced into each container. These are the concentrations used in the assay: 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, and 0.03125 mg/mL. Three replicates were used on each concentration.

For the negative control, (5) mL of artificial seawater was used and for the positive control, one (1) mL of dimethyl sulfoxide (DMSO) was added to four (4) mL of artificial seawater to make it 5 mL. Using pure 100% DMSO as a positive control can have a detrimental effect on cells. A concentration of 5% is considered excessively high and can result in the dissolution of cell membranes. The cytotoxicity of DMSO is dependent on its concentration, with the highest concentrations being toxic to cell lines (Sangweni *et al*., 2021). The containers were left uncovered under the lamp. The number of surviving shrimps was counted and recorded after twenty-four hours (Supraja *et al*., 2018; Waghulde *et al*., 2019; Fauziah *et al*., 2022) and the percentage of mortality was calculated using Equation 2. The toxicity of the extract or fractions was assessed by determining the $LC_{50}$ using the probit method.

Equation 2. The formula used to calculate the percentage of mortality:

$$\% \text{ of death larvae} = \frac{\text{Number of dead larvae}}{\text{Total number of initial larvae}}$$

**Chorioallantoic membrane (CAM) assay**

For the CAM assay, duck eggs were used and this experiment is a well-established *in vivo* and *in ovo* animal model widely utilized to examine tumor biology in various applications. (Lokman *et al*., 2020).

Fertilized duck eggs (*Anas domisticus*) were purchased from a local vendor and the eggs were carefully wiped clean to remove dirt and increment. Eggs were incubated before administering the *D. meyeniana* plant extract group and the control group. The candling method was performed to ensure duck egg viability.

Positive and Negative control groups were used in this study. 1 mg/mL concentration of

Celecoxib was used as a positive control. Celecoxib can inhibit angiogenesis (Rosas *et al*., 2014; Roa *et al*., 2017). This control was administered to the eggs. For the Negative control, no administering is done, leaving the egg as it is.

The fertilized eggs were incubated from day one until day eight at 37 °C and 70% humidity. Before injecting the plant extract, the eggs were disinfected with 70% ethanol to eliminate unwanted pathogens. The air chamber of the egg was located, and a small hole was punched to insert the needle into the syringe. A 0.3 mL test substance was injected through the hole using the syringe. The treated eggs were sealed with parafilm once the test substance was administered entirely to the egg, and incubated for 48 hr (Ribatti *et al*., 2006).

On the 48$^{th}$ hour of incubation, treated and control groups of eggs were carefully opened, exposing the CAM, and a hole was created enough to fit the suction of a one cc syringe. The albumin was removed using the syringe; around 5 mL or more of albumin was removed from the egg membrane to lower down. The bottom part of the egg was sealed using parafilm. A rectangular shape was drawn, measuring 3 × 2 cm around the embryo's area. The hard shell was carefully removed, exposing the embryo. A clear photo was taken on the CAM with blood vessels. A dissecting microscope was used to locate the primary blood vessels. Image J was used to quantify the picture taken from the duck egg.

**Visual assessment and photography**

The CAM was examined at the application site for angiogenesis. The CAM vascularity can be expressed as a percent inhibition formula. The CAM vascularity percent inhibition formula:

$$\% \text{ inhibition} = \frac{N - N_0}{N} \times 100$$

Where N= Negative Control and $N_0$= Treatment.

**Data analysis**

The data gathered in this study was subjected to statistical analysis using SPSS, GraphPad Prism 9 software, and Microsoft Excel. One-way analysis of variance (ANOVA) was conducted to determine the significant difference in the number of branches present in the chorioallantoic membrane among the six treatments used in the study followed by Tukey's test used to analyze significant differences among the treatment group and control group. A *p-value* of <0.05 was defined as statistically significant.

## RESULTS AND DISCUSSION

**Phytochemical screening of the crude extract**

The phytochemical screening result shows the presence of saponins, flavonoids, alkaloids, and steroids. Table 1 shows levels of abundance to represent detected bioactive chemicals.

**Table 1.** Phytochemical screening of the methanolic crude extracts of *Dendrocnide meyeniana* indicating its phytochemical constituents with its level of detection, high detection (+++), moderate detection (++), low detection (+), and no detection (-)

| Phytochemicals | Level of detection |
|---|---|
| Saponins | +++ |
| Flavonoids | ++ |
| Alkaloids | ++ |
| Steroids | + |
| Tannins | - |
| CN-Glycosides | - |
| Anthraquinones | - |

Natural plant products can be utilized as anti-angiogenic medicines, replacing synthetic medications that are costly or toxic, restricting their usage in many patients. Natural plant compounds were identified with anti-angiogenic qualities and the molecular processes by which these molecules exert their anti-angiogenic effects (Lu *et al*., 2016).

Our result shows that there is a high detection level of saponins, a moderate detection level for flavonoids and alkaloids, and a low level for Steroids. Majnooni *et al.* (2023) have shown that plants high in saponins have long been a staple in human diets for medicinal and general health advantages.

Saponins are polar molecules of one or more sugar chains attached to a triterpene or steroid aglycone. Additionally, they have a wide range of industrial uses in the food, cosmetics, and pharmaceutical industries (Osbourn *et al*., 2011). Saponins as one of the largest groups of natural compounds, have shown notable anti-angiogenic effects in treating several cancers *in vitro* and *in vivo* (Khan *et al*., 2022; Majnooni *et al*., 2023).

## Brine shrimp lethality test of the plant extracts using different solvents

The $LC_{50}$ values are categorized based on Clarkson's Toxicity Criteria, which categorizes extracts as non-toxic if their $LC_{50}$ value is above 1000 ppm, low toxic if the $LC_{50}$ value falls between 500-1000 ppm, medium toxic if it ranges from 100-500 ppm, and highly toxic if the value is between 0-100 ppm (Clarkson *et al*., 2004) (Figure 3 & Table 2).

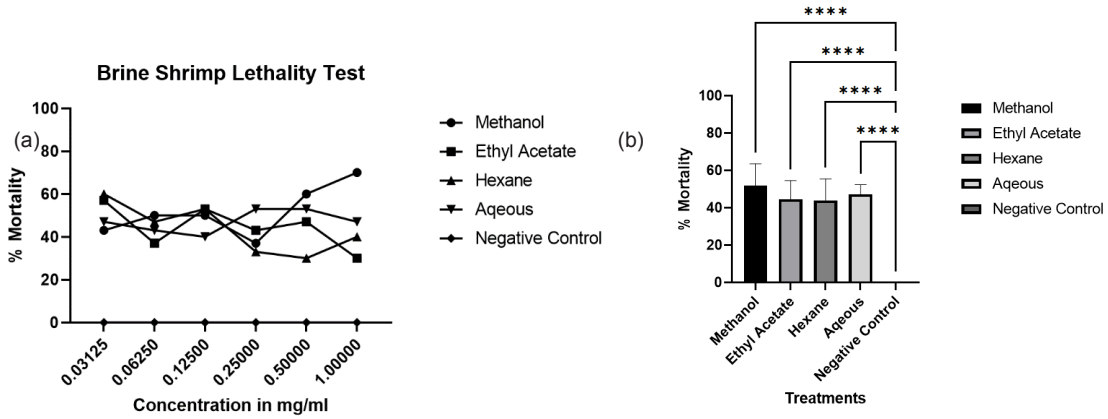


**Fig. 3. (a)** Percent mortality values of the different plant extracts in six different concentrations. **(b)** Bar graph showing the percent mortality per treatment and *p*-values less than or equal to 0.05 where **Significant at 1% level; ***Significant at 0.1%; **** Significant at 0.01%.

**Table 2.** $LC_{50}$ values of the different plant extracts in Parts Per Million (PPM) show different toxicity classifications as shown in the (+) sign. Their value (+++) is the highly toxic, (++) for medium toxic, and (+) for low toxic

| Extract | $LC_{50}$ | Toxicity |
|---|---|---|
| Methanol | 136.49 | (++) |
| Ethyl acetate | 52.98 | (+++) |
| Hexane | 75.51 | (+++) |
| Aqueous | 657.81 | (+) |

## Chorioallantoic membrane assay

The CAM assay used the most active extracts of *D. meyeniana* to test their anti-angiogenic potential on eight-day-old duck eggs. The concentrations of ethyl acetate and hexane (0.125 mg/mL & 0.25 mg/mL ) were determined based on the brine shrimp lethality assay, indicating medium toxicity. Each treatment had five egg replicates, and the anti-angiogenic activity was evaluated by analyzing branch points.

The results showed that hexane demonstrated 34.07% inhibition after 48 hr, and hexane at 0.25 mg/mL displayed the highest inhibition rate of 34.98% shown in Figure 4. Ethyl acetate at 0.125 mg/mL exhibited 15.93% inhibition after 48 hr of incubation, while ethyl acetate at 0.25 mg/mL showed 19.05% inhibition shown in Figure 4. The positive control (Celecoxib) achieved 72.08% inhibition. In the comparison of treatments, both concentrations of ethyl acetate were significantly different from the positive control at a 1% level, and the positive and negative controls were significant at a 0.1% level. Therefore, the hexane partition exhibited a higher inhibition rate compared to ethyl acetate. These results suggest that both ethyl acetate and hexane possess anti-angiogenic properties and can inhibit blood vessel formation.

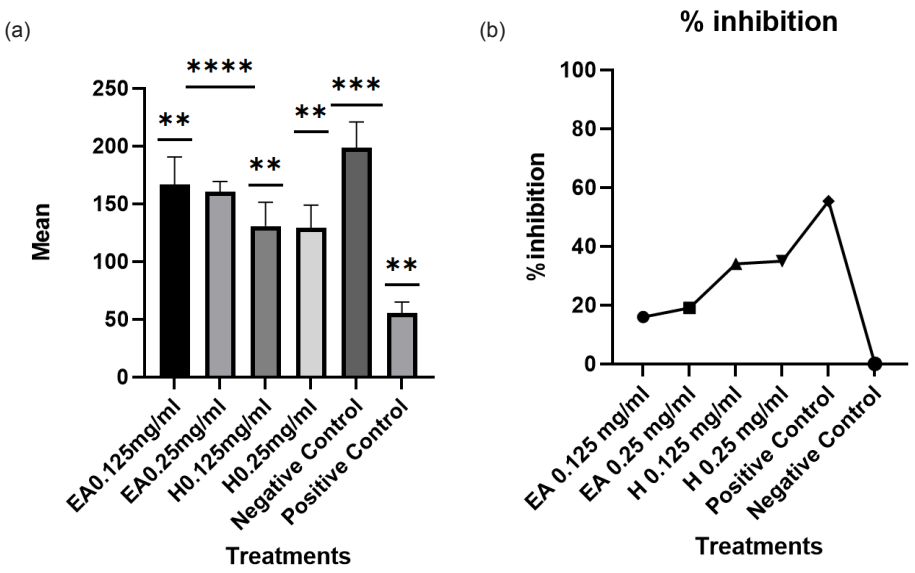


**Fig. 4.** Comparison of means by treatment. ** significant at 1% level; *** significant at 0.1%. (b) Percent inhibition anti-angiogenic property of ethyl acetate (EA) and hexane (H) at 125 ppm and 250 ppm, respectively.

Numerous substances have been documented to activate and hinder the formation of blood vessels in the CAM assay. These substances encompass growth factors, hormones, naturally occurring compounds, medications used for cancer treatment, gases, organo-metallic compounds with pro-angiogenic properties, antibiotics, antibodies, and artificially synthesized small compounds (Ribatti, 2016). Results of this study showed that the duck eggs exposed to a hexane fraction of the plant extract have fewer branch growths than the negative control shown in Figure 5. Of the two hexane concentrations, the 0.25 mg/mL concentrations show the least blood vessel formation with an inhibition percentage of 34.98%. While the 0.125 mg/mL of hexane plant crude extract appears to have slightly more visible blood vessels formed than the 250 ppm, with an inhibition percentage of 34.07%. Both concentrations of ethyl acetate, have less percent inhibition compared to the two hexane concentrations and the positive control as shown in Figure 6. This implies hexane at 0.125 mg/mL and 0.25 mg/mL has an anti-angiogenic property given the percent inhibition. The ethyl acetate concentrations have less percent inhibition though still showing an anti-angiogenic property.

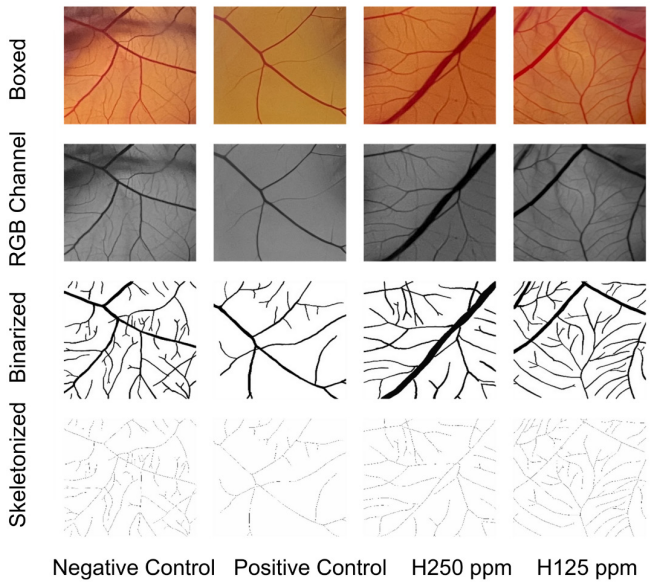


**Fig.5 .** Sample images from Fiji (ImageJ) software analysis. Image processing was done to quantify the number of branching points for each replicate. Left side from top to bottom: Individual boxed images (900x900), RGB Channel,bBinarized, and skeletonized for analysis. From left to right, negative control, positive control, and hexane concentrations were H250 ppm (0.25 mg/mL) and H125 ppm (0.125 mg/mL), respectively.

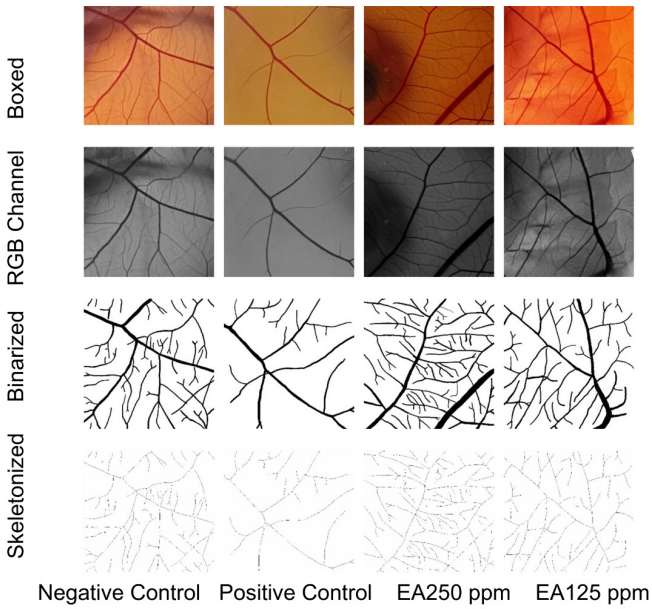


**Fig. 6.** Sample images from Fiji (ImageJ) software analysis. Image processing was done to quantify the number of branching points for each replicate. Left side from top to bottom: Individual boxed images (900x900), RGB channel, binarized, and skeletonized for analysis. From left to right, negative control, positive control, and ethyl acetate concentrations were 250 ppm (0.25 mg/mL) and 125 ppm (0.125 mg/mL), respectively.

## CONCLUSION

Phytochemical screening found saponins, flavonoids, alkaloids, and steroids from *Dendrocnide meyeniana* which are the basis for having medicinal properties for certain diseases. In conclusion, the root extract of *Dendrocnide meyeniana* has anti-angiogenic properties based on the inhibition percentages of the partitions.

## ACKNOWLEDGEMENT

The authors wish to thank the Department of Science and Technology-Advance Science and Technology Human Resource Development (DOST-ASTHRDP) for the financial support of EMDT. We would also like to thank the Department of Research of Mindanao State University-Iligan Institute of Technology (MSU-IIT) SO#00148-2022 for financial support.

## ETHICAL STATEMENT

Not applicable.

## CONFLICT OF INTEREST

All authors have declared no conflict of interest.

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