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# *In Vitro* Anti-angiogenic Activity of the Ethanolic Extract of *Mentha arvensis* Linn. (Yerba Buena) Leaves Using Duck Chorioallantoic Membrane (CAM) Assay

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**ABSTRACT —** The *M. arvensis* Linn. is known to have beneficial pharmacological and antioxidant properties, but its anti-angiogenic activity remains unexplored. The study aimed to determine the anti-angiogenic activity of the ethanolic extract of *M. arvensis* Linn. leaves using *Anas platyrhynchos domesticus* L (duck) chorioallantoic membrane (CAM) assay. Fertilized *A. platyrhynchos domesticus* L. eggs were treated with different concentrations (1 mg/mL, 0.5 mg/mL, 0.1 mg/mL, and 0.01 mg/mL) of the *M. arvensis* ethanolic extract, and blood vessel inhibition was assessed. The extract's effects were compared to negative - Normal Saline Solution (NSS) and positive (Methotrexate) controls. The results exhibited an anti-angiogenic activity based on the number of secondary blood vessels ($p \leq 0.05$), 1 mg/mL (*mean* = 32, *sd* = 31), 0.5 mg/mL (*mean* = 41, sd = 31), 0.1 mg/mL (*mean* = 39, *sd* = 38), and 0.01 mg/mL (*mean* = 50, *sd* = 32). This was compared to the negative control (450 mg/500 mL NSS) (*mean* = 303, *sd* = 125). The anti-angiogenic activity of the different concentrations was comparable to one another and to the positive control (Methotrexate) (*mean* = 73, *sd* = 68). The best anti-angiogenic result was seen at 1 mg/ml of the crude ethanolic extract. This study supports the potential development of cost-effective, plant-derived anti-angiogenic agents that may benefit fields such as oncology and pharmaceutical development, subject for further study.

*Keywords: Mentha arvensis Linn.,* anti-angiogenic activity, CAM assay, ethanolic extract

## I. Introduction

Angiogenesis is the biological process of forming new blood vessels from pre-existing capillaries and post-capillary venules (Jung *et al.*, 2007). This process needs to be highly regulated because too much blood vessel growth can have detrimental effects. Diseases linked to excessive angiogenesis include retinopathy, liver cirrhosis, psoriasis, and cancer (Bisht *et al.*, 2010). Since angiogenesis plays an important role in these conditions, blocking is a promising treatment strategy. Moreover, while synthetic angiogenesis inhibitors exist, they are costly, and resistance to these drugs has been reported (Loges *et al.*, 2010). Because phytomedicines generally have fewer side effects, there is an increased interest in discovering natural anti-angiogenic compounds with anti-angiogenic properties (Chinsembu *et al.*, 2010).

The Philippine Department of Health (DOH) approved ten herbal medicines recommended for human use as home remedies, including Yerba buena *(M. arvensis Linn.)* plant as described by the Philippine Institute of Traditional and Alternative Health Care. However, its other potential medicinal uses, such as anti-angiogenic effects, remain underexplored. *M. arvensis Linn.)* was chosen in the study because it has a strong antioxidant activity (Abd El-Rahman *et al.*, 2016). Antioxidants lower reactive oxygen species (ROS), which are critical in the pathologies of angiogenesis-dependent diseases as they initiate the formation and growth of blood vessels (Hassan *et al.*, 2014). Antioxidants, thus in part, contribute to the inhibition of angiogenesis. A strong link between potent antioxidants and anti-angiogenesis was identified by a number of studies suggesting that natural products with antioxidant activity may also have anti-angiogenic effects (Li *et al*., 2016).

This study uses the chorioallantoic membrane (CAM) of the duck embryo. The CAM model is low cost, simple, and highly reliable. In comparison with other animal models, it retains compounds longer because there is no excretion, allowing the use of minute

amounts of experimental compounds (Lokman *et al.*, 2012). 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 (Parsons-Wingerter *et al.*, 1998). Quantification of blood vessels in large amounts of CAM models can be used to screen drugs from sample plant extracts (Wang *et al.*, 2004).

In CAM assay, methotrexate is used as a positive control. Methotrexate is a folic acid antagonist used to treat cancer, inflammatory, and lung diseases (Maksimovic, *et al.*, 2020). In cancer therapy, it prevents the proliferation of malignant cells while inhibiting angiogenesis (Cronstein, 1997). Blood vessels become dense around the applied anti-angiogenic test compound or even disappear.

The potential uses of the *M. arvensis Linn.* need further exploration. This study examined its anti-angiogenic activity using the CAM assay. The result of this study opened new therapeutic opportunities, especially for angiogenesis-dependent diseases like cancer, aiming to help reduce their associated morbidity and mortality in the future

## II. MATERIALS AND METHODS

### Data Collection Procedures with Parameters to Measure

***Identification of M. arvensis Linn.***

*M. arvensis Linn.* was verified through a certification issued by the Department of Agriculture (DOA) – Bureau of Plant Industry. The plant is not a listed taxon. Preliminary identification was based on the reference book by Dr. Eduardo Quisumbing (1978).

**Figure 2.**
*Mentha arvensis Linn.*

This figure displays *Mentha arvensis Linn.*, commonly known as Yerba buena, a plant widely recognized for its medicinal properties. Its leaves are characterized by their elliptic to oblong-ovate shape, measuring 1.5 to 4 centimeters in length, and having a strong aromatic scent. They are short-stalked, with toothed margins and a rounded or blunt tip. The plant features prostrate, smooth, and much-branched stems, typically purplish in color, extending up to 40 centimeters in length, with terminal branches that ascend. The flowers are hairy, purplish to bluish, and are arranged in axillary head-like whorls. The calyx teeth are triangular or lanceolate and hairy, while the corolla is also hairy. *M. arvensis Linn.* is not classified as endangered, threatened, or extinct; it is commonly found in many regions and is considered a species of least concern.

*M. arvensis Linn.* belongs to the Kingdom Plantae, Phylum Angiosperm, Class Magnoliopsida, Subclass Lamiidae, Order Lamiales, Family Lamiaceae, Genus *Mentha*, and Species *Mentha arvensis.* Its phytochemical properties tested positive for alkaloids, anthraquinones, flavonoids, glycosides, and tannins, while it tested negative for saponins, steroids, and triterpenoids.

***Collection of M. arvensis Linn.***

Mature green leaves of *M. arvensis Linn.* were collected in Alegre, Oton, Iloilo. The plants were harvested before 6 AM to avoid contamination from feeds and insects, at least three days after rainfall, and at a distance of 100 meters away from the road.

***Preparation of M. arvensis Linn. Leaves Ethanolic Extract***

Fresh samples of the leaves of *M. arvensis Linn.* were carefully and thoroughly washed with tap water and drained for approximately 30 minutes. These were then air-dried. Afterward, the air-dried plant was macerated in ethanol for 24 hours at room temperature to make the *M. arvensis Linn.* ethanol leaf extract at 1:10 (w/v) concentrations. Extraction was carried out at room temperature. The liquid extract was separated from the residue by filtration using a muslin cloth, followed by Whatman filter paper. A rotary evaporator was used to obtain the concentrated ethanol extract (Manaharan *et al.*, 2014).

***Collection of Anas platyrhynchos domesticus L. Eggs***

Three-day-old fertilized *Anas platyrhynchos domesticus L.* eggs were collected from Alegre, Oton, Iloilo. Dirt, feathers, and excrements were carefully removed from the exterior of the eggs using distilled water and were sanitized with 70% ethyl alcohol. Eggs weighing at least 50 g were chosen. Egg candling was performed in order to determine if the eggs were fertilized. To ensure

accuracy, this process was validated by a professional. An egg is confirmed fertilized if a tiny speck on the yolk called the germ spot is present during egg candling. Fertilized eggs were incubated for a day for acclimatization at a relative humidity of 60-62% and temperature of 37°C (Domaub *et al.*, 2024).

***Sterilization of equipment***

All materials were sterilized in an autoclave at 121°C for 15 minutes at 15 psi, both before and after use, to maintain accuracy and ensure the safety of the experimental procedures.

***Preparation of Test Samples***

The crude ethanolic extracts were dissolved in water to yield a stock solution of 30 mg/3 mL. Serial dilutions were then performed to obtain concentrations of 1 mg/mL, 0.5 mg/mL, 0.1 mg/mL, and 0.01 mg/mL.

***Chorioallantoic Membrane (CAM) Assay***

The test specimens were divided into six treatment groups: one group for each concentration of the crude ethanolic extract, one group for the negative control, and one group for the positive control. After one day of acclimatization, the eggs were wiped individually with 70% ethanol. After the day of incubation, 3-4 mL of albumin were removed to allow detachment of membrane from the eggshell. The eggs were inoculated with the treatments using a syringe, injecting 0.1 mL into the fertilized *Anas platyrhynchos domesticus L.* egg embryo through the air sac. The shells were resealed using parafilm to prevent dehydration and contamination, then further incubated for 24 hours in preparation for post-test observation and documentation.

The fertilized eggs were carefully cracked open and placed in a petri dish for examination after the incubation period. Using a stereomicroscope, images of the chorioallantoic membrane (CAM) were captured. Quantification of the vascular network was performed by measuring the degree of vascularization in each treatment group. The angiogenic effects were assessed by counting the number of blood vessels in a defined area using the AngioTool software, a reliable and widely used image analysis program designed to quantify vascular structures from microscopic images (Zudaire *et al*., 2011).

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

X-Y x 100

X

where X is the number of blood vessels in 450 mg/500 mL Normal Saline Solution (negative control) and Y is the number of blood vessels in the treated CAM.

**Data Analysis/Statistical Treatment**

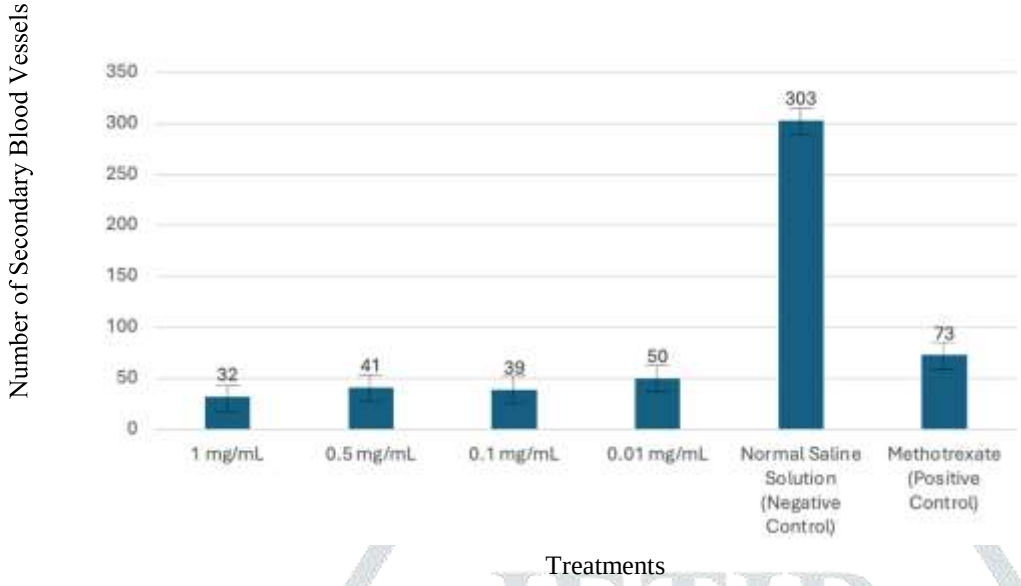
***Statistical Tools***

The number of blood vessels for each treatment was quantified using the AngioTool software and expressed as mean ± standard deviation (*sd*). The quantitative data obtained were subjected to statistical analysis. The differences among the treatments were compared using One-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test. They were considered significant at $p \leq .05$. The statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) edition 17.0.

## III. RESULTS AND DISCUSSIONS

The *M. arvensis Linn* ethanolic leaf exhibited an anti-angiogenic activity based on the number of secondary blood vessels ($p \leq 0.05$). The 1 mg/mL (*mean* = 32, *sd* = 31), 0.5 mg/mL (*mean* = 41, sd = 31), 0.1 mg/mL (*mean* = 39, *sd* = 38), and 0.01 mg/mL (*mean* = 50, *sd* = 32). This was compared to the negative control (450 mg/500 mL NSS) (*mean* = 303, *sd* = 125). The anti-angiogenic activity of the different concentrations was comparable to one another and to the positive control (Methotrexate) (*mean* = 73, *sd* = 68). The best anti-angiogenic result was seen at 1 mg/ml of the crude ethanolic extract (Figure 4).

**Figure 4**




*Note.* Figure 4 shows the average number of secondary blood vessel in the different concentrations the ethanolic extract of *M. arvensis Linn.* Leaves and the negative and positive control.

The results of the Duck Chorioallantoic Membrane (CAM) assay demonstrate an anti-angiogenic effect of crude ethanolic extract of *M. arvensis Linn.* leaves at various concentrations (1 mg/mL, 0.5 mg/mL, 0.1 mg/mL, and 0.01 mg/mL).

The anti-angiogenic effect of the crude ethanolic extract of *M. arvensis Linn.* leaves are likely due to its bioactive compounds, such as flavonoids, and phenolic compounds, which have been reported to possess anti-inflammatory and antioxidant properties (Jadhav *et al*., 2016). These compounds may interfere with vascular endothelial growth factor (VEGF) signaling pathways, which are critical for new blood vessel formation (Niu & Chen, 2010). Flavonoids, for instance, have been documented to down regulate VEGF expression and block the activation of its receptor VEGFR-2, thereby reducing endothelial cell proliferation and migration (Li *et al*., 2016). Phenolic compounds also contribute by neutralizing reactive oxygen species (ROS), which are known to stimulate angiogenic signaling (Hassan *et al*., 2014). The strongest inhibition at 1 mg/mL suggests that a higher concentration delivers sufficient bioactive compounds to significantly suppress angiogenesis. The comparatively higher number of blood vessels observed at lower concentrations may be attributed to the diminished availability of bioactive compounds, which, while still exerting an inhibitory effect. The compounds may not be present in sufficient amounts to produce the same extent of angiogenesis suppression as seen at higher concentrations.

The comparison with Methotrexate supports this interpretation, as Methotrexate is a known angiogenesis inhibitor (Maged *et al.*, 2019). All trials show comparable performance between the highest concentration of *M. arvensis Linn.* and the positive control, indicating its promising potential as a natural angiogenesis inhibitor.

The previous study by Jadhav *et al.* (2016) *in vivo* demonstrated the treatment of 500 ug dose of ethyl acetate extracts of *M. arvensis Linn.* caused marked inhibition of neovascularization. In this study, the crude ethanolic extracts at concentrations (1 mg/mL, 0.5 mg/mL, 0.1 mg/mL, 0.1 mg/ mL, and 0.01 mg/mL) showed also inhibitory effects on blood vessels formation antiangiogenic in vitro. Both of the research studies concluded that the crude ethanolic extract and ethyl acetate of *M. arvensis Linn.* leaves showed antiangiogenic activity.

The study was limited by the use of only the crude ethanolic extract and lacked analysis of individual bioactive compounds. Additionally, only short-term observations were made, which might not fully capture delayed angiogenic responses. The anti-angiogenic activity of the crude ethanolic extracts of *M. arvensis Linn.* leaves were true only when the plant was collected from Alegre, Oton, Iloilo, Philippines.

This study supports the use of *M. arvensis Linn.* as a potential source for anti-angiogenic agents. Given its traditional use and accessibility, it may pave the way for cost-effective, natural therapeutic options in diseases like cancer, diabetic retinopathy, and psoriasis where angiogenesis plays a central role. This can be verified through future studies.

## IV. CONCLUSION

The results of the study demonstrated that the crude ethanolic extract of *M. arvensis Linn.* leaves exhibited anti-angiogenic activity as observed through the duck chorioallantoic membrane (CAM) assay. Among the tested concentrations (1 mg/mL, 0.5 mg/mL, 0.1 mg/mL, and 0.01 mg/mL) the 1 mg/mL concentration showed the highest inhibitory effect on blood vessel formation, in relation to the negative (450 mg/500 mL Normal Saline Solution) and positive control (1.25 mg/500 mL Methotrexate). When compared to

the negative control (450 mg/500 mL Normal Saline Solution), all concentrations exhibited a reduction in angiogenesis, suggesting that the extract possesses anti-angiogenic properties. Furthermore, all concentrations of the ethanolic extract showed a comparable inhibitory effect when compared to the positive control (1.25 mg/500 mL Methotrexate). The study supports the development of cost-effective, plant-derived anti-angiogenic agents that may benefit fields such as oncology and pharmaceutical development. Future studies are needed to isolate, characterize, and quantify the individual bioactive compounds responsible for the observed effects.

## V. RECOMMENDATIONS

Despite the fact that this research supports the anti-angiogenic potential of *M. arvensis Linn.*, future research should use image analysis software to quantify all vessel branches (primary and tertiary) as this study only measured secondary vessels. It would be beneficial to expand on the present findings by testing a wider range of extract concentrations, which may help determine the minimum effective dose and allow further exploration of the potential effects and limitations of higher concentrations. It is also recommended to isolate and analyze the compounds in *M. arvensis Linn.* that are responsible for its anti-angiogenic effects, as through studying these compounds to better understand how *M. arvensis Linn.* works as an angiogenesis inhibitor.

## VI. ACKNOWLEDGMENT

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