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Phytochemical Screening and Evaluation of Angiogenesis Activity of *Hyptis Capitata* Jacq. Leaf Ethanolic Extract

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ABSTRACT

Plants are valuable compounds that can be used to develop medicines against various diseases. *Hyptis capitata* Jacq. is a shrub commonly found on roadsides and is used to treat ailments by local people. In this study, the ethanolic leaf extract of *H. capitata* was subjected to phytochemical screening to determine the secondary metabolites present in the plant leaves. Chorioallantoic membrane assay (CAM) was used to evaluate the plant extract's antiangiogenic activity. Concentrations of 0.1 ppm, 1 ppm, 10 ppm, 100 ppm, 1000 ppm, and 10000 ppm from the plant's leaf extract, distilled water as the negative control, and retinoic acid as the positive control were treated on the CAM. Results revealed that the *H. capitata* leaves contain steroids, flavonoids, saponins, and tannins. The plant's leaf extract has antiangiogenic potential as 1000 ppm and 10000 ppm concentrations significantly decreased the blood vessels ($P<0.05$) with percent inhibition of 26.78 % and 35.49 %, respectively. Studies exploring other assays for angiogenesis and toxicity tests to support the plant's potential as an angiogenesis inhibitor are recommended.

Keywords: *Ethnomedicine, Chorioallantoic Membrane Assay*

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1 Introduction

Angiogenesis is a physiological process of forming new blood vessels from preexisting vascularization. This process is necessary for wound healing growth, development, and granulation tissue formation. However, it also plays a fundamental role in pathological conditions such as chronic inflammation, tumor growth, and metastasis (Salas and Totaan 2015). Thus, angiogenesis inhibitors can be a potential approach to the abnormal growth of the blood vessels that may lead to these pathological diseases (Camposano et al. 2016).

Chorioallantoic membrane (CAM) assay has been utilized to perform *in vivo* angiogenesis or anti-angiogenesis studies (Naik et al. 2018). It is a well-established model that provides a vascular structure that can be used to study tumor cell invasion, angiogenesis/anti-angiogenesis, and metastases (Mousa et al. 2017). CAM model is advantageous because it is relatively simple, low cost, highly reproducible, and is a closed system composed of multilayer ectoderm with extracellular matrix proteins that mimic the physiological cancer cell environment (Ribatti 2017; Lokman et al. 2012). Besides angiogenesis and tumor invasion, CAM assay can also be used to study bone regeneration (Moreno-Jimenez et al. 2016) and avian body growth (Rosal et al. 2020; Gamallo et al. 2016), among others.

Plants are a valuable source of medication

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and play a significant role in global health (Sofowora et al. 2013). It is estimated that 80% of the world's population relies on herbal medicine as primary health care, especially in developing countries. Incorporating herbs in traditional medicine practices is an intrinsic part of the culture in those communities (Ekor 2014). According to Oladeji (2016), plant-derived medications have been identified through recent studies of curative, therapeutic, traditional remedies, and most notably, indigenous people's folk knowledge. Despite recent advances in science and technology, some of these claims and beliefs remain irreplaceable.

Hyptis capitata Jacq., is a slightly aromatic herbaceous plant belonging to the family Lamiaceae and is a common weed found in open fields and along roadsides (Sulistyaningsih et al. 2017). The decoction of leaves of *H. capitata* has been used for cough, malaria, diarrhea, stomachache, newborn baby care, fever, gas pain, and flatulence. Crushed leaves are used for cuts and wounds by Manobo of Agusan (Dapar et al. 2020). Despite the various ethnomedicinal uses of *H. capitata*, this plant needs more scientific studies, particularly on its antiangiogenic activity.

This study aims to determine some of the active classes of secondary metabolites and evaluate the effects of *H. capitata* leaf crude ethanolic extract on the angiogenesis and morphometrics of the duck *Anas platyrhynchos* embryos. The result of this study will be additional knowledge on *H. capitata* and serves as a scientific basis for alternative and natural angiogenesis inhibitors.

2 Materials and Methods

Plant collection and extraction

The *H. capitata* plant samples were collected from Buenavista, Agusan del Norte (8°57'43.54" N, 125°23'51.09" E), and species authentication was done the Biology Department of Caraga State University (CSU), Ampayon, Butuan City. The plant extraction was carried out following the standard method described by Guevarra et al. (2005). The collected leaves were washed thoroughly and air-dried for one week. The dried *H. capitata* leaves were cut into smaller pieces and placed in ziplock bags for further extraction. The cut pieces of the air-dried plant were subjected to ethanolic extraction in the CSU Chemistry Laboratory. Briefly, the 300 grams of the cut-dried leaves were soaked in 1500 mL of 95% aqueous ethanol for 48 hours at room temperature with intermittent shaking. The extracts were filtered through an ordinary filter paper and concentrated using a rotary evaporator to yield crude ethanolic crude extract.

Phytochemical screening

The phytochemical screening for secondary metabolite determination was done following the methods described by Guevarra et al. (2005). Briefly, the test for alkaloids was done using Dragendroff's test, the test for steroids using Liebermann-Burchard test, the test for flavonoids using Based-Smith and Metcalf Method, the Froth test for saponins, and ferric chloride test for tannins.

Preparation of test treatments and CAM assay

The chorioallantoic membrane assay was performed thrice following the protocol described by Ribatti (1997) and Vergara et al. (2021) with few modifications. Working concentrations of the *H. capitata* leaf ethanolic extract were set to explore the concentration-dependent angiogenic effects on duck embryos. In this study, concentrations of the leaf extract were 0.1, 1, 10, 100, 1000, and 10000 ppm, respectively. Since distilled water was utilized as the negative control and solvent to dissolve the stock solution of the leaf extracts, retinoic acid was used as the positive control. Both negative and positive controls were used to compare the effects of leaf extract solutions in the *in ovo* experiment.

Eight-day-old fertilized duck (*A. platyrhynchos*) eggs were bought from a local supplier in Ampayon, Butuan City, Philippines. Eggs were thoroughly cleaned by removing dirt and excrement and were sanitized by wiping 70% ethanol to the surface of the eggs. All the eggs that were used were incubated at 37°C. Three fertile eight-day duck eggs were used for each experimental group, and the experiment was conducted thrice.

Maintained sterile conditions, and appropriate labeling was strictly followed. To ensure that the eggs were fertilized, egg candling was performed to observe if there was a germ spot on the yolk. Unfertilized eggs were discarded, and the fertilized ones were used for downstream analyses.

A window in the eggshell, about 1 x 1 cm, was

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made to expose the chorioallantoic membrane for experimental manipulation. A 100 µL of *H. capitata* leaf solutions and controls was topically applied on the CAM of each egg and sealed using sterile parafilm tape. Eggs were incubated for 72 h at 37°C.

Visual assessment and photography

On day 11 post-fertilization, eggs were harvested by reopening the sealed portion, and shells were removed to expose the CAM widely. Each CAM was photographed twice, which was used for counting the blood vessel vascularity. Branch points were manually calculated using Fiji software (https://fiji.sc). The CAM inhibition was expressed as a percentage of the control as follows:

$$Vascular\ Inhibition = \frac{BP_c - BP_t}{BP_c} x100\%$$

Where BPc is the number of branch points of the negative control while BPt is the number of branch points of the treatment.

Morphometric analysis

Weight and morphometry of the embryos of eggs treated with the test chemicals were measured following the method described by Vergara et al. (2021) to test if the test concentrations of the plant extract may play a role in the development of the duck embryo. The same concentration of extracts and control treatments in the CAM assay was used in the morphometric analysis. The following indices measured were the eye diameter (ED), crown-to-rump length (CRL), head-beak length (HBL), forelimb length (FL), and hindlimb length (HL). The weight of the embryos was measured using a digital weighing scale, and morphometric indices were measured using a Vernier caliper.

Statistical analysis

The results were presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by the Tukey post-hoc test was utilized to compare between groups. Differences with $P<0.05$ between experimental groups were considered statistically significant. The statistical test used Statistical Package for the Social Sciences (SPSS) version 23.

3 Results and Discussion

Phytochemical constituents in H. capitata leaf extract

Phytochemicals play an essential role in the growth and development of plants (Martinez et al., 2017). It helps protect plants from unfavorable environmental conditions; hence, it attracts interest and has been utilized in various studies to evaluate its beneficial effects on acute and chronic human diseases (Kadioglu et al. 2013). In this study, the leaf ethanolic extract of *H. capitata* was subjected to phytochemical screening to determine the secondary metabolites. *H. capitata* contained steroids, flavonoids, saponins, and tannins. No alkaloids were observed in the test (Table 1).

Steroids are significant medicinally active organic compounds. It was reported to have cholesterol-reducing properties, which can lower up to 15% of cholesterol levels, act as cancer preventative, and has significantly been marketed as a dietary supplement (Sultan 2015). Flavonoids are a group of phenolic compounds mainly found in fruits and vegetables and have strong antioxidant properties against oxidative stress (Rodríguez-García et al. 2019; Sahib et al. 2010). It also exhibited other biological activities, including antibacterial, antiviral, anti-allergic, anti-inflammatory, and anticancer (Chaves et al. 2020; Kopustinskiene et al. 2020; Abotaleb et al. 2018). According to Ravishankar et al. (2013), flavonoids in the process of tumorigenesis or carcinogenesis interfere with cancer progression by modulating the signal transduction pathways limiting the proliferation, angiogenesis, and metastases.

Saponins are primarily seen in plants and have been studied for various properties (Faizal and Geelen, 2013) because of their pharmacological activities such as anti-inflammatory, antibacterial,

Table 1. Result of phytochemical screening of *Hyptis capitata* leaf ethanolic extract.

Phytochemicals	Presence/ Absence
Alkaloids	absent
Steroids	present
Flavonoids	present
Saponins	present
Tannins	present

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antiviral, antifungal, anticancer, and cytotoxic activity (Ashour et al. 2019). Tannins, another phytoconstituent abundant in plant new leaves and flowers, possess various biological functions. Hydrolyzable tannins were reported to have anticancer, antiangiogenic, antioxidant, anti-inflammatory, and anti-ulcer activities (Amarowicz and Janiak 2019). The absence of alkaloids may be associated with the environment where the plant was collected, affecting the plant's phytochemicals (Kusuma et al. 2020).

Antiangiogenic activity

The antiangiogenic potential of leaf ethanolic crude extract of *H. capitata* was evaluated using the CAM assay (Figure 1). CAM assay revealed a significant decrease in vascular density (Figure 2A) and a substantial increase in vascular inhibition (Figure 2B) as the concentration of the plant extract increased ($P<0.05$). However, post-hoc analysis showed that only the positive control ($P=0.013$), 1000 ppm ($P=0.04$), and 10000 ppm ($P=0.002$) have a significant difference compared to the negative control.

The highest concentration, 10000 ppm, has the highest vascular inhibition of 35.49%. It was followed by 1000 ppm with 26.78 % inhibition, while 0.1 ppm had the least percent inhibition of 16.75%. The result of the CAM is consistent with the study of Mamutuk & Usman (2017) on the antiangiogenicity of *Hyptis suaveolens*, which revealed a dose-dependent effect on angiogenesis. However, the IC50 revealed higher than the highest concentration, 10000 ppm, applied on the CAM.

The inhibition of angiogenesis is possibly done by suppressing the expression of vascular endothelial growth factor or VEGF, matrix metalloproteinases, and inhibiting the migration and proliferation of endothelial cells (Subbaraj et al. 2021). VEGF is considered one of the most important pro-angiogenesis factors. It enhances the blood vessels' permeability while altering the extracellular matrix-degrading enzyme production, which causes the vascular system to expand. It also activates endothelial cells through its receptors, leading to the secretion of metalloproteinases that will allow the migration of endothelial cells (Hoseinkhani et al. 2020; Niu and Chen 2010). Notably, plant compounds such as flavonoids and tannins have been reported to target angiogenesis by inhibiting the expression of VEGF (Kadioglu et al. 2013). The presence of these phytochemicals may explain the anti-angiogenic effect of *H. capitata* ethanolic leaf extracts.

Morphometric analysis of the embryos

Avian embryos can be used for the toxicity assessment of compounds (Smith et al. 2012).

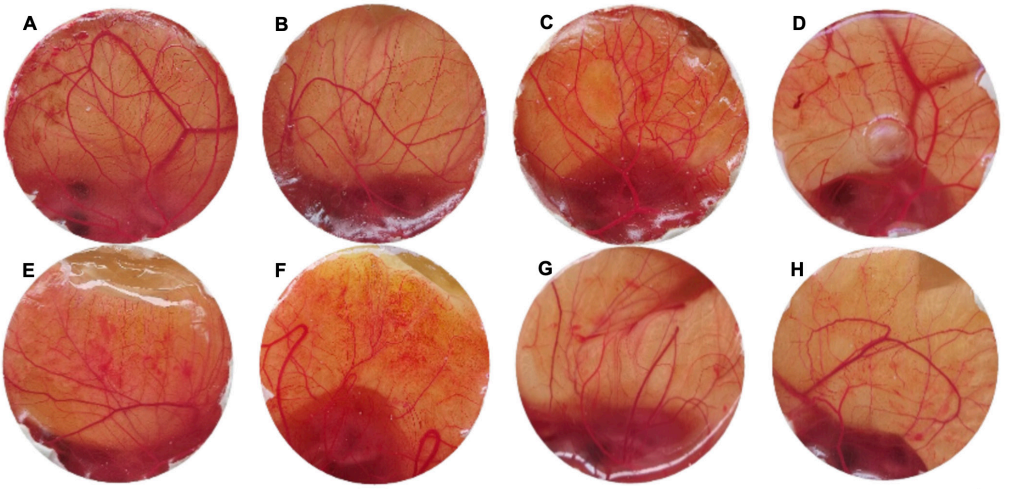

Figure 1. Representative of duck CAM treated with distilled water (a) and retinoic acid (b) control treatments and *Hyptis capitata* leaf ethanolic extract with the concentrations: 0.1 ppm (C), 1 ppm (D),10 ppm (E),100 ppm (F), 1000 ppm (G), and 10000 ppm (H).

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Morphometric analysis was done to determine the effect of *H. capitata* leaf ethanolic extract on the development growth of the duck embryos. Morphological parameters such as CRL, HBL, FL, HL, and ED were measured in the analysis.

There were no abnormalities in the gross appearance and form of the embryos treated with controls and different concentrations of the plant's leaf ethanolic extract. No significant effect was found on the weight, CRL, HB, FL, HL, and ED upon treatment with the various concentrations of the plant extract (Table 2).

Treatments administered topically to the CAM may reach systemic circulation and alter the embryo's development (Ribatti 2017). However, the current result contradicts prior short-term (48-72 h) teratogenicity studies conducted to explore the effects of compounds on the avian system's embryonic development (Al-Qahdi et al. 2019; Gamallo et al. 2016; Baharara et al. 2014). The resistance of the morphometries to the inhibitory effects may be due to the small number of bioactive compounds exerted by the plant extract (Mamutuk and Usman 2018). This result may suggest that the *H. capitata* leaf ethanolic extract may not be potent enough to disrupt the normal development of the duck embryos.

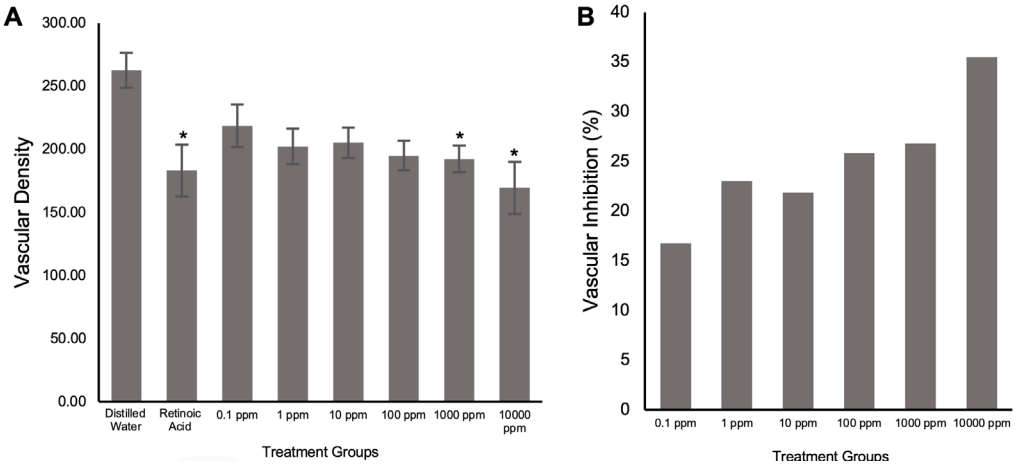

Figure 2. Blood vessel density (A) and vascular inhibition (B) of CAM treated with control treatments and different concentrations of *Hyptis capitata* leaf ethanolic extract.

**Significantly different from the negative control based on Tukey's posthoc test (P<0.05)*

Table 2. Means (± SEM) of weight, crown-rump (CRL), head-beak (HBL), forelimb (FL), hind limb (HL) length, and eye diameter (ED) duck embryos treated with controls and different concentrations of *Hyptis capitata* leaf ethanolic extract.

	Weight (g)	CRL (mm)	HBL (mm)	FL (mm)	HL (mm)	ED (mm)
Distilled Water	1.36 ± 0.17	34.62 ± 3.43	14.97 ± 1.12	8.96 ± 1.19	10.93 ± 0.77	5.45 ± 0.26
Retinoic Acid	1.29 ± 0.17	34.13 ± 2.13	13.56 ± 1.81	8.62 ± 1.24	10.96 ± 0.84	5.75 ± 0.37
0.1 ppm	1.27 ± 0.34	33.34 ± 3.75	13.31 ± 2.19	8.68 ± 2.01	11.00 ± 1.87	5.09 ± 2.12
1 ppm	1.40 ± 0.17	34.57 ± 1.38	14.48 ± 1.32	8.997 ± 1.31	11.85 ± 1.00	5.92 ± 0.53
10 ppm	1.35 ± 0.18	34.67 ± 1.53	14.50 ± 1.30	9.72 ± 1.27	11.80 ± 1.18	5.85 ± 0.65
100 ppm	1.30 ± 0.25	34.23 ± 2.64	14.39 ± 2.07	9.59 ± 1.57	12.09 ± 1.59	5.96 ± 0.42
1000 ppm	1.35 ± 0.12	34.82 ± 2.27	13.62 ± 1.20	9.27 ± 1.49	11.85 ± 1.36	6.01 ± 0.42
10000 ppm	1.27 ± 0.13	32.95 ± 2.42	13.48 ± 1.49	8.62 ± 1.34	11.05 ± 1.29	5.74 ± 0.57

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4 Conclusions and Recommendations

The current findings revealed the presence of steroids, flavonoids, saponins, and tannins in the leaf ethanolic extract of *H. capitata*. The plant extract can cause significant concentration-dependent inhibition on the blood vessels of the *A. platyrhynchos* chorioallantoic membrane. However, the extract did not significantly affect the embryo's weight, CRL, HBL, FL, HL, and ED. With this, it is recommended for future research to use higher concentrations of the plant extract and explore the effects of long-term exposure to the extract in other higher animal model organisms.

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5 Statement of Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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