ANTI-ANGIOGENICITY AND TERATOGENICITY OF *HYPTIS SUAVEOLENS* LEAF ETHANOLIC EXTRACT IN MALLARD DUCK (*ANAS PLATYRHYNCHOS*) EMBRYOS

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ABSTRACT: *This study was conducted to evaluate the anti-angiogenic potential and teratogenic effect of the ethanolic extract from Hyptis suaveolens leaves, through chorioallantoic membrane (CAM) vascularity and teratogenicity assays in mallard duck (Anas platyrhynchos) embryos. Each of the seven treatments namely, 0.01%, 0.06%, 0.1%, 1% and 5% extracts, alpha-tocopherol (positive control) and pure olive oil (negative control), was randomly administered by injection into the air cell of 8-day old embryos that were incubated at approximately 37.5º C. On the 12th day of incubation, five embryos in each treatment were examined for CAMs vascular growth. In a 3 by 3 cm representative fractal segment of each CAM, the primary, secondary and tertiary blood vessels were counted, and the diameters of primary and secondary blood vessels were measured. On the 18th day of incubation, the remaining alive embryos in each group were examined for gross external morphological abnormalities. Results showed that the number of tertiary blood vessels and the total number of blood vessels were significantly reduced. However, the diameter of blood vessels, and the body weight, body length, body mass index and lengths of eye diameter, beak, neck, forelimbs and hind limbs to body length ratio of the treated embryos were not significantly reduced. The results revealed that the tested five concentrations of H. suaveolens leaves contain components that are anti-angiogenic but are not teratogenic. Therefore, it warrants further studies as a potential source of herbal medicine and drugs for anti-angiogenic cancer therapy.*

Keywords: Anti-angiogenicity, Teratogenicity, CAM Assay, *Hyptis suaveolens*, *Anas platyrhynchos*

1. INTRODUCTION

In 1996 there were 10 million new cancer cases worldwide and six million deaths attributed to cancer, and in 2020 there are predicted to be 20 million new cases and 12 million deaths [1]. This life threatening effect of cancer can be attributed to its ability to grow and spread to distant organs.

Tumors can only grow up to 2-3 mm through diffusion from the nearby blood vessels [2]. In order to expand, tumor cells secrete growth factors (e.g., vascular endothelial growth factor or VEGF) to acquire their own blood vessels, thereby acquiring enough oxygen and nutrients, in a process called angiogenesis [3]. Tumor cells can then metastasize, exiting from the blood circulation, establishing a new colony at distant site, and depending on angiogenesis for survival [4]. This lead to the foundation of antiangiogenic cancer therapy-the prevention of new vessel sprouts from penetrating into early tumor implant. It has been validated as an effective cancer treatment for a growing number of cancer types, including colorectal, renal, liver, lung, brain, pancreatic neuroendocrine tumors (NET), gastrointestinal stromal tumors (GIST), multiple myeloma, and myelodysplastic syndrome [5].

Chorioallantoic membrane (CAM) assay is one of the cheaper and more reproducible assays used in monitoring the efficacy of drugs [6]. The rich vasculature of the CAM is useful in observing and quantifying the effects of pro- and anti-angiogenic compounds [7]. In addition, teratogenicity assay is the measure of the teratogenic effect (developmental defects) of a certain drugs to the embryo [8].

As synthetic techniques improved, it became possible to create new medicinal compounds based on natural products. *Hyptis suaveolens*, a rigid sweetly aromatic herb belongs to the family Lamiaceae or Labiatae is a common weed distributed throughout the tropics and subtropics [9], and is very abundant in open places at low and medium altitude throughout the Philippines [10]. It is commonly called as bush mint in English, suob-kabayo in Filipino and M'ngyak in Meranao. *H. suaveolens* has been reported to possess antioxidant, anti-inflammatory, antimicrobial, anti-diarrheal, antihelmintic, anti-diabetic, anticancer, wound-healing and insecticidal properties [11]. The leaves from this plant are used as a substitute for infusion in tea in West Africa, as a "bate" or memory soup in India, as a stomachic in Indonesia, and as antispasmodic, anti-rheumatic and antisoporific in Philippines [12].

With this knowledge, the present study was conducted to evaluate the anti-angiogenicity and teratogenicity of *H. suaveolens* using chorioallantoic membrane (CAM) vascularity and teratogenicity assays.

2. EXPERIMENTAL DETAILS

2.1. Plant Collection and Identification

M'ngyak (*H. suaveolens*) plants were collected from Balo-i, Lanao del Sur, Philippines and were identified taxonomically based on Merill's Flora of Manila [13] with the help of Prof. Fatimah M. Natangcop of the Department of Biology, MSU Main Campus, Marawi City. The collected plants were washed thoroughly with tap water to remove dirt. Fresh leaves without any signs of microbial contamination were carefully selected out from the whole plants, cut into small piece, and air-dried through the hanging technique to remove excess water.

2.2. Preparation of Treatments

The ethanolic plant extraction employed was based on the methodology described by Guevara et al. (2005) [14]. Before extraction, the dried leaves were ground using a blender. About 400 grams of the ground leaves were soaked in a prepared 95% ethanol solution for 72 hours, followed by

filtration. The filtered extracts obtained were then concentrated to a volume of 15 ml in a rotary evaporator at 45-50 degree Celsius. The concentrated extract was diluted to 0.01%, 0.06%, 0.1%, 1.0%, 5.0% concentrations, with pure olive oil as solvent. Ten ml of each concentration were stored in a properly labeled test tubes covered with cotton plugs. Pure olive oil and alpha-tocopherol were used as the negative and positive controls, respectively.

2.3. Collection of Experimental Eggs

A total of 140 one-day old *A. platyrhynchos* embryos were obtained from a licensed commercial supplier at Pala-o, Iligan City, Philippines.

2.4. Treatments of Embryos

The *A. platyrhynchos* embryos were subjected to injection of treatments after 8th day of incubation. Prior to this, the viability of embryos were determined through candling of individual eggs using a candler lamp. A viable egg contains visible living embryo, heartbeat and massive blood vessels, whereas non-viable egg has a blood ring. To prevent bacterial contamination, viable eggs were cleaned with 70% ethanol using cotton swab.

Upon administration of treatments, a tiny hole was made at the air space of the egg using an 18-gauge size tuberculin needle. A volume of 0.2 ml of each treatment was injected in the air cell using a 1 cc tuberculin syringe. To prevent the inner membrane of the egg from breaking, only a very little part of the needle was inserted using different syringes per concentration; aseptic technique was employed to prevent contaminations; after inoculation, the opening was carefully sealed with melted paraffin wax using a spatula. The eggs were then returned to the incubator. Every 24 hours, the egg viability was monitored. In calculating the percent mortality (PM), equation 1 was used.

$$PM = \frac{\text{(Number of dead individuals)}}{\text{(Total number of sample population per group)}} \times 100 \quad (1)$$

2.5. Chorioallantoic Membrane (CAM) Assay

The CAM assay was performed according to the method followed by Ribatti et al. (2006) and Raga et al. (2013), with some modification from (Goling, 2014) [15-17]. On the 12th day incubation, the remaining eggs were placed on their lateral sides to position the CAM and the embryo. Out of the 90 surviving embryos, 35 embryos (five replicates per treatment) were randomly selected for CAM assay. The paraffin seal was removed. The CAMs were harvested by removing the hard shell leaving intact the soft membrane covering the embryo. These were carefully placed in a sterilized glass with the aid of a spatula. The CAMs were photographed using a Sony 10.1 mega pixels camera and were examined using Image J software version 1.49s by Rasband (2015) [18].

In counting the primary, secondary and tertiary blood vessels and in measuring the diameter of the primary and secondary blood vessels, a representative fractal segment of 3 by 3 cm for each CAM was examined. To assert consistency, the primary blood vessel nearest to the heart was used as the reference point for each fractal segment. The largest blood vessel from the heart is designated as the primary blood vessel (PBV); secondary blood vessels (SBV) are those blood vessels branching out from the primary blood vessel; tertiary blood vessels (TBV) are those blood vessels branching out of the secondary blood vessel [19]. The Percent vascularity inhibition (PVI) was calculated using equation 2 [20].

$$PVI = \frac{\text{(N of CAM treated by X - N of CAM treated by Plant Extract)}}{\text{(N of CAM treated by Negative Control)}} X100 \quad (2)$$

Wherein, N = Total vessel number

2.6. Teratogenicity Assay

The remaining alive treated eggs were examined on the 18th day of incubation. The body weight and body length were recorded to calculate the Body Mass Index (BMI) in mg/mm2. The embryos were examined for the presence of any gross morphological anomalies in terms of the lengths of the eye diameter, beak, neck, forelimbs and hind limbs. The mean values of the left and right measurements of the eye diameter, forelimb and hind limb were further calculated. To remove the influence of different body lengths (due to biological factors), all measurements were divided over the body length of the examined embryo, thereby getting their ratio. Digital weighing scale was used in determining the body weight and Image J software version 1.49s by Rasband (2015) was also used in measuring the length.

2.7. Experimental Design and Statistical Analysis

The type of the treatment and the replicate number were randomly drawn using a paper. The eggs were marked with a labeling tape. This technique was repeated without replacement until all eggs were assigned.

The data measured were expressed as mean and were analyzed using One-way Analysis of Variance (ANOVA) and Kruskal Wallis test followed by Tukey HSD as post hoc test for pairwise comparison of the differences between the experimental and control duck embryos. P values less than or equal to 0.05 are considered as indicative of significance. The statistical tests were conducted using the SPSS Version 14.0 with the help of Prof. Sony M. Magno, statistician of the Institute of Science Education, MSU Main Campus, Marawi City, Philippines.

3. RESULTS AND DISCUSSION

3.1. Chorioallantoic Membrane (CAM) Assay

The anti-angiogenic activity of *H. suaveolens* leaf ethanolic extract in *A. platyrhynchos* embryos is shown in Table 1 and Fig. 1. The reduction of the number and diameter of blood vessels was considered as positive indications of anti-angiogenic potential.

In all examined fractal segments, only one PBV, and either two or three SBV were observed. In terms of TBV and total number of blood vessels (PBV + SBV + TBV), CAM of embryos treated with 5% extract displayed the lowest mean number, whereas those treated the negative control displayed the highest. Kruskal – Wallis test of both data revealed that the effects exerted by the different treatments were highly unequal (P=0.000). The result shows that as the concentration of *H. suaveolens* extract is increased, the number of blood vessels is decreased.

Table 1. Summary of mean values of the number of primary (PBV), secondary (SBV), tertiary blood vessels (TBV), the total number of blood vessels and the diameter of PBV and SBV per 3 by 3 cm representative fractal segment of CAM of treated duck embryos

Treatment	Number of Blood Vessels			
	PBV	SBV	TBV	TOTAL
0.01% extract	1.0	2.2	7.6a	18.00a
0.06% extract	1.0	2.0	7.4a	17.33 a
0.1% extract	1.0	2.0	6.4a	15.66 a
1% extract	1.0	2.2	6.2a	15.66 a
5% extract	1.0	2.0	4.8a	13.00 a
Positive Control	1.0	2.0	8.2a	18.66 a
Negative Control	1.0	2.4	18.0b	35.66b
P value		**0.309**	**0.004***	**0.004***

a. Mean values within columns followed by the same letter are not significantly different at P≤0.05 by Tukey's HSD test.
b. The P values were computed using Kruskal-Wallis test.

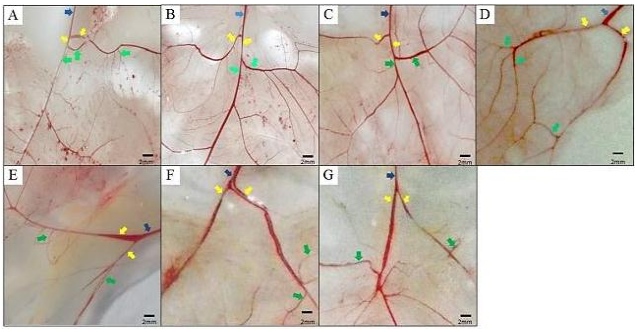

Figure (1) Representative photographs of CAM vascular area of *A. platyrhynchos* embryos treated with pure olive oil (negative control) (A), alpha-tocopherol (positive control) (B), 0.01% (C), 0.06% (D), 0.1% (E), 1% (F), and 5% (G) *H. suaveolens* leaf ethanolic extracts. Arrows indicate primary (blue); secondary (yellow); and tertiary (green) blood vessels.

Percent vascularity inhibition (PVI) was calculated to determine the degree of inhibition of blood vessel formation [20] exerted by *H. suaveolens* (Table 2). Apparently, the entire test extracts have much higher inhibitory rate compared to those embryos treated with the positive control.

Table 2. Summary of mean values of the diameter of primary blood vessels (PBV), and secondary blood vessels (SBV) and the Percent vascularity Inhibition (PVI) per 3 by 3 cm representative fractal segment of CAM of treated duck embryos

Treatment	Diameter of Blood Vessels (mm)		PVI
	PBV	SBV	
0.01% extract	0.435	0.341	49.533
0.06% extract	0.504	0.356	51.402
0.1% extract	0.480	0.340	56.075
1% extract	0.519	0.341	56.075
5% extract	0.521	0.357	63.551
Positive Control	0.510	0.362	47.664
Negative Control	0.561	0.378	0
P value	**0.628**	**0.977**	

a. The P values were computed using One-way ANOVA.

The diameters of primary and secondary blood vessels are presented in Table 2. Accordingly, PBV of CAMs of embryos treated with 0.01 % extract and SBV of those embryos treated with 0.1% displayed the smallest mean diameter. In both cases, those treated with the negative control had the largest mean diameter. However, one-way ANOVA of both data showed no significant difference (P = 0.196 and 0.826, respectively). This implies the plant extract's inability to reduce the transverse growth of PBV and SBVs in the CAMs of duck embryos.

Angiogenesis is essential in tumor growth and metastasis as the process provides necessary oxygen and nutrition for the growing tumor [21]. Quantitative analysis of the present study revealed that all embryos treated with the different concentrations (0.01%, 0.06%, 0.1%, 1%, and 5%) of ethanolic extract of *H. suaveolens* leaves significantly inhibited the outgrowth of new blood vessels (TBV and the total number of blood vessels) in the chorioallantoic membrane of *A. platyrhynchos* embryos. Embryos treated with 0.01% extract (56.31%) and those treated with both 1% and 5% extracts (53.40%) show higher percent PVI compared with those treated with alpha-tocopherol (49.51%). These observations indicate the anti-angiogenic potential of *H. suaveolens* leaves.

Alpha-tocopherol, is one of the eight isoforms of Vitamin E: α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol [22]. Beyond their primary antioxidant function, alpha-, gamma- and delta tocopherols can also reduce inflammatory angiogenesis in human microvascular endothelial cells [23]; the effect could be due to the suppression of vascular endothelial growth factor (VEGF) from human tumor cells [24]. This correlates with the result in this study, wherein the positive control significantly inhibited the formation of blood vessels with respect to those treated with the negative control.

Preliminary phytochemical screening of ethanol extracts of *H. suaveolens* leaves revealed the presence of alkaloids, glycoside, saponin, tannins and flavonoids as major active constituents [25]. Additionally, colorimetric methods of aerial parts of *H. suaveolens* methanolic extract revealed its phenolic antioxidant components: flavonoids content (lg quercetin equivalents/mg extract) of 28.58 ± 1.74 and total phenols content (lg gallic acid equivalents/mg extract) of 74.56 ± 1.33 [26].

In support of the potential anti-angiogenic activity of *H. suaveolens*, other studies have demonstrated that selected phytochemical constituents extracted from this plant were found to inhibit angiogenesis. Generally, phenolics are antioxidant [27]. Interestingly, in a study made by Pil´atov´a et al. (2010), flavonoids are also anti-angiogenic through a variety of mechanisms: inhibiting vascular endothelial growth factors (VEGF) expression, inhibiting endothelial cell migration, and decreasing matrix metalloproteinases [28]. The flavonoid present – quercetin, which is normally found in apples, cherries, citrus fruits, raspberries, red grapes, broccoli, onion and leafy greens, has been shown to have an anti-angiogenic property [29]. According to Davis et al. (2010), as cited by Li et al. (2011), its antiangiogenic properties include inhibition of MMP-2 and MMP-9 secretion from tumor cells and inhibition of endothelial cell proliferation and migration.

Moreover, a correlation between antioxidant and anti-angiogenesis was observed when Yasuda et al. (1999), as cited by Piaru et al. (2012), reported that a vital endogenous

ROS, hydrogen peroxide, plays a major role in stimulating angiogenesis *in vitro* [30, 27]. Antioxidants have the ability to scavenge reactive oxygen species or ROS (e.g., superoxide, hydrogen peroxide, and hydroxyl radicals) that may cause damage to DNA, proteins and lipids [31-32]. Too much ROS give rise to oxidative stress, promoting a variety of human ailments such as atherosclerosis, hypertension, inflammation and cancer. Likewise, as concluded by Prabakaran et al. (not dated), the anti-angiogenic property of *Ceropegia pusilla* extracts might have been due to preventing signaling of angiogenic agent from epithelial cells, or by induction of apoptosis through free radical scavenging mechanism, which prevents promotional events with CAM tissue [33].

To date, there have been extensive studies on natural product compounds and extracts that showed potent anti-angiogenic activity, in conjunction to having good antioxidant activities [34]. Apparently, Gavani and Paarakh (2008) showed that leaves of *H. suaveolens* methanolic extract exhibited strong antioxidant radical scavenging activity with LC50 value of 14.04µgmL-1, comparable to those obtained for gallic acid and BHA (0.4 and 1.15µgmL-1) [35]. We can therefore assume that the anti-proliferative activity of *H. suaveolens* extract in this study may arise from its anti-oxidative capacity, primarily due to the presence of flavonoids.

This observed vascular inhibitory effect of *H. suaveolens* suggests its anti-cancer property. In fact, anti-angiogenic therapy is a novel method of treating cancer, primarily because it is not directed towards killing cells (as in chemotherapy, which kills both the cancerous and healthy cells), but rather targets the abnormal growth of blood vessels that supply nutrients and oxygen to the solid mass of tumors. This mechanism will prevent and/or stop the growth and spreading to distant location of tumor cells [36]; a fundamental function that is highly comparable to the use of cytotoxic drugs, which are limited by a number of factors, such as toxicity, tumor resistance and lack of targeted cell death. Though, according to Wu and his colleagues (2008), there are some anti-angiogenic agents that show variety of side effects, including thrombosis, leukopenia, lymphopenia, and immunomodulation [37]. Therefore, major advances have done in the field of angiogenesis, including the elucidation of the signaling pathways of several endogenous angiogenesis factors, and the discovery of several natural and synthetic angiogenesis stimulators and inhibitors, leading to the translation of experimental drugs into clinical use [38].

In the present study, there is a slight decrease in the diameter of primary and secondary blood vessels of embryos treated with the different concentrations of *H. suaveolens* leaves ethanolic extract. However, statistical analysis of the data revealed that these are not significant, thereby indicating that the anti-angiogenic potential of the plant extract and the positive control do not include the capacity of reducing the diameter of blood vessels.

The percent mortality of duck embryos after the administration of treatments showed a lower rate of 45% that was observed for embryos treated with 0.1% and 1% concentrations, relative to those treated with the negative control and positive control, which exhibited the same mortality rate of 55%. Both the negative and positive controls displayed the same mortality rate of 55%. No dead embryos were recorded after 24 and 48 hours. Dead embryos were observed only after 72 hours of incubation. There was no specific trend observed. This indicates the extract's low embryogenic toxicity despite its anti-angiogenic activity. Some external factors might have also caused the mortality in all treatments, such as the individual biological variations among the embryos (e.g. individual absorptive capacities and genetic differences of the embryos), the size and abnormal positioning of eggs during incubation, and the improper handling of eggs.

3.2. Teratogenicity Assay

Table 3. Summary of the mean values of the morphological parameters measured in duck embryos treated with the test extracts after 18 days of incubation.

Treatments	Body Weight (g)	Body Length (mm)	BMI (mg/mm^2)
0.01% extract	8.150	73.910	1.494
0.06% extract	10.50	82.261	1.553
0.1% extract	8.460	78.492	1.368
1% extract	10.60	85.112	1.459
5% extract	9.167	77.049	1.554
Positive Control	8.650	74.627	1.576
Negative Control	8.900	81.656	1.334
P value	0.121	0.265	0.277

a. The P values were computed using One-way ANOVA.

The teratogenic activity of *H. suaveolens* leaf ethanolic extract in *A. platyrhynchos* embryos is shown in Table 3. Embryos treated with 0.06%, 1% and 5% extracts displayed higher body weights as compared to those treated with pure olive oil and alpha-tocopherol; those treated with 0.06% and 1% extracts have higher body lengths compared to the negative control; and those treated with 0.06% and 5% extracts exhibited the highest BMI. However, One-way ANOVA of these data showed that the differences are not significant (P = 0.121, 0.265 and 0.277, respectively).

All tested concentration of *H. suaveolens* leaf extracts showed significantly the same eye diameter, neck, forelimb and hind limb lengths, with the negative control, except on beak length. One-way ANOVA of the data on beak length revealed that the effects exerted by the different treatments were highly significant at P = 0.004; embryos treated with the 0.01% extract displayed the highest mean beak length.

Analysis of the data showed that the test extract did not significantly affect all the morphological parameters being measured at all concentration, relative to the effects exerted by the negative control. In fact, the test extract at 0.01% concentration resulted to a significantly higher beak length. Beak development starts on the 4th day of incubation, and hardening starts on the 10th day of incubation. Further lengthening takes place approximately until the 18^{th} day of incubation. It is possible that beak embryonic cells were able to resist the inhibitory effect exerted by the plant extract since the bioactive components are present in small amounts only. Moreover, a high P value doesn't give us specific trend on the increasing concentration of test extract. In order to confirm this, a test using more replicates should be done.

Table 4. Summary of the mean values of the morphological parameters measured in duck embryos treated with the test extracts after 18 days of incubation.

Treat-ments	Eye Diame-ter	Length (mm)			
		Beak	Neck	Forelimb	Hind limb
0.01% extract	0.112	0.181a	0.231	0.381	0.471
0.06% extract	0.100	0.149cd	0.219	0.351	0.420
0.1% extract	0.103	0.166bc	0.250	0.368	0.405
1% extract	0.100	0.155cd	0.250	0.359	0.455
5% extract	0.104	0.161c	0.240	0.353	0.428
Positive Control	0.109	0.156cd	0.226	0.364	0.415
Negative Control	0.107	0.159c	0.223	0.365	0.431
P value	0.195	**0.004***	0.085	0.369	0.061

a. Mean values within columns followed by the same letter are not significantly different at P≤0.05 by Tukey's HSD test.
b. The P values were computed using One-way ANOVA.

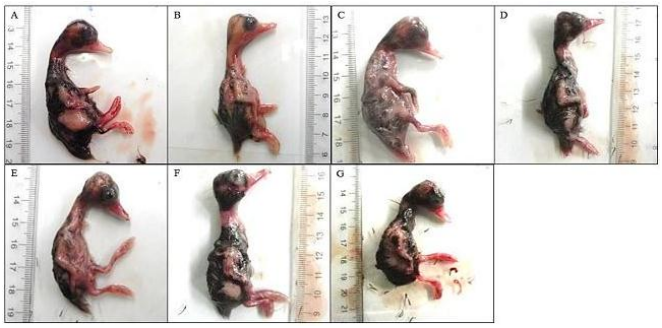

Figure (2) Representative photographs of *A. platyrhynchos* embryos treated with negative control (A), positive control (B), 0.01% (C), 0.06% (D), 0.1% (E), 1% (F) and 5% (G) *H . suaveolens* leaf ethanolic extracts at 18th day of incubation.

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4. CONCLUSION

The present study reports the inhibition of angiogenesis by the ethanolic extract of *H. suaveolens* leaves in mallard duck embryos. The effect is dose-dependent at 0.01%, 0.06%, 0.1%, 1% and 5% concentrations. The leaf extract does not significantly affect the body weight, body length, body mass index, eye diameter, and lengths of beak, neck, forelimbs and hind limbs. Therefore, *H. suaveolens* may provide a new source of chemical agents for anti-angiogenic cancer therapy and warrants further studies.

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