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Antiangiogenic Activity of Oregano *(Origanum Vulgare)* Aqueous Extract In-Vivo Through Chick Chorioallantoic Membrane (Cam) Assay

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ABSTRACT

Angiogenesis is the formation of new blood vessels and is required in tumor growth, metastasis and cancer progression. Philippine traditional medicine preparations have shown a comprehensive and function- regulating characteristics. Oregano (Origanum vulgare) is an annual succulent herb which distributed throughout the world. Currently, there have been some related reports on the preventive effects on chronic diseases such as cancer and heart diseases with oregano. The study aimed to evaluate the antiangiogenic activity of oregano crude leaf extract through Chick Chorioallantoic Membrane (CAM) assay. The density and length (per branching) of the blood vessels were measured through a microscope and ToupView software calibrated using a micrometre respectively. The researchers analyzed the data gathered using one-way Analysis of Variance (ANOVA), post hoc tests were also performed using Duncan Multiple Range Test (DMRT) to confirm the results of the ANOVA. Statistical results in the mean blood vessel density and length of blood vessels among the treatments which is at .001 and .000 respectively is lesser than the 0.05 level of significance which rejects the null hypothesis. DMRT results confirm the significant differences among the treatments. The results indicate that the crude leaf extract of Origanum vulgare significantly reduced the length and density of the blood vessels of chick chorioallantoic membrane, indicating its angiogenesis inhibiting capabilities. These results suggest that antiangiogenic effects of crude leaf extract of Origanum vulgare may be mediated via its antioxidant and anticancer effects.

Keywords: Antiangiogenesis; cancerous tumours; CAM assay; tumour growth; oregano leaf extract; in-vivo

INTRODUCTION

Cancer is a debilitating condition that causes millions of deaths per year. There are more than 14.1 million new cases on cancer, over 8.2 million deaths due to cancer and there are 32.6 million individuals worldwide living with cancer was reported in 2012. Cancer causes cells to abnormally multiply which then forms tumors that metastasizes and spreads to neighboring healthy cells if left untreated. Most prevalent treatments, specifically chemotherapy and radiotherapy, while proven efficient are very costly and have underlying negative health effects.

Although in recent years, immense progress has been made in our understanding of molecular mechanisms and cellular regulation of angiogenesis in important diseases like cancer, clinical development of antiangiogenic agents for the therapy of cancer remains challenging. Since solid tumors account for more than 85% of cancer mortality in humans, tumor growth and metastasis are dependent on blood vessels. Therefore, nowadays, targeting tumor angiogenesis is one of the most widely studied areas to find new therapeutic strategies. In screening potential drug candidates against angiogenesis, a broad range of plant products were screened for antiangiogenic effects (Elluru et al., 2009; Mojzis et al., 2008; Liu et al., 2008; Oner et al., 2007).

Angiogenesis is a complex biological process that occurs normally in development, turnover and remodeling of mature vascular networks (Bryan and D'Amore, 2007; Guran et al., 2004). Angiogenesis is the formation of new vessels by endothelial sprouting, that is, endothelial cell migration, proliferation and tube formation. Angiogenesis is useful in some cases such as tissue infarcts when oxygen necessity increases; conversely in some cases it may be harmful (Staton et al.,2006; Tufan and Satıroglu-Tufan, 2003). By the surrounding neoplastic cells increasing excessively and without control, it may cause the tumor to be nourished and oxygenated and thus encourage the growth of the tumor.

Han and Parker (2017) conducted a research study where they assessed the anti-inflammatory, tissue remodeling as well as

immunomodulatory, and anticancer abilities of Origanum vulgare essential oils through human skin disease mode. In their research, it was mentioned that the oregano essential oil contains a high content of carvacrol which has been largely used and studied for its antioxidant, antibacterial, antifungal, anticancer, and anti-inflammatory properties. (Han and Parker, 2017). The aforementioned phytochemical component of the plant with its anticancer capabilities can possibly have a significant impact on its capability to inhibit angiogenesis.

In the study conducted by Sri Devi et al., 2013 on the angiogenic inhibiting property of the *Leucas aspera* plant, they applied the widely used Chick Chorioallantoic Membrane (CAM) assay to study the angiogenic activity of the said plant. This study aimed to assess the antiangiogenic activity of the oregano leaf extract through chick chorioallantoic membrane assay or CAM assay in terms of blood vessel density and length of blood vessels.

In the research conducted by Salas and Totaan, 2015 on the antiangiogetic ability of extracts coming from selected Philippine herbal plants, it was revealed that between the three selected herbal plants: sabungai (*Gynura nepalensis*), pandan (*Pandanus odoratissimus L.*) and, tsaang-gubat (*Carmona retusa)Masam*, sabungai is more effective in inhibiting angiogenesis followed by pandan then tsaang-gubat.

On the other hand, it is proven that in the various parts of the *Calotropis procera* in the research of Kalimuthu, Prabakaran and Saraswathy 2014 on the *Antiangiogenic Properties Stem, Leaf and Flower Extracts of Calotropis Procera in Chick Chorioallantoic Membrane (CAM),* that the methanolic leaf extracts showed the higher angiogenic activity followed by methanolic flower extract. Thus, medicinal plants have been usually and broadly used long before the advancement of technology and numerous researches that have been conducted provide proof of its efficiency.

The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane that acts as a gas exchange surface and its function is supported by a dense capillary network. Due to its extensive vascularization and easy accessibility, the CAM has been broadly used to study the morphological function of the angiogenesis process *in vivo* and to investigate the efficacy and mechanisms of pro-angiogenic and anti-angiogenic molecules.

The CAM angiogenesis assay is performed by implanting a membrane or coverslip containing the compound of interest on the chick embryo chorioallantoic membrane through a hole cut in the egg shell. Subsequently, the CAM is fixed and the blood vessels are quantified by counting the number of blood vessel branch points. The main advantages of CAM model are high vascularization properties, high reproducibility, simplicity, and cost effectiveness.

The province of Abra is rich of plants that have medicinal properties, such as *Origanum vulgare* locally known as oregano. *Origanum vulgare* from the family *Lamiaceae*, is now extensively distributed all throughout the world. Based on the scientific researches, oregano extracts and its constituents have shown consistently antimicrobial activities against food-borne pathogens. Thus, it also contains antioxidants which are potent that might have benefits toward the nervous and cardiovascular system, The protocol followed by the Acebedo 2014 on "Egg Windowing and the Chorioallantoic Membrane Assay" was implemented on this study with minimal modifications. The chick eggs were incubated at 37°C while altering the position of the eggs during incubation relieves symptoms of an inflammation, and it also modulates blood sugar and lipids (Singlatery, 2010).

Most people who are diagnose or inspected with cancer cannot afford the therapies and treatments that they seriously need. Hence, the continuous research for the development of affordable and equally efficient alternatives. The use of oregano leaf extract for treatment of cancer has not been rigorously evaluated. In this study, leaves of oregano were extracted, and systematic study of its antiangionenic mechanism systematically examined. The objective is to give a theoretical basis for the therapeutic use of the oregano leaf extract in inhibiting angiogenesis on chorioallantoic membrane of a fertilized egg in terms of density and length of its blood vessels with the use of Chick Choroallantoic Membrane (CAM) assay.

METHODOLOGY

Research Design

This research study used the complete randomized design (CRD). Each treatment was replicated three times and from each replicate, ten blood vessels were observed, measured and recorded, comprising to 30 blood vessels observed per treatment. Required permits were acquired as well as strict ethical protocols were noted prior to the conduct of the investigatory project.

A. Acquisition and Incubation of Chicken Eggs

The material for the acquisition of chicken eggs was an egg tray to hold the eggs in place while windowing and the 16-capacity incubator as the chamber of controlled environment for the hatching was used for the incubation of the chick eggs.

The researchers acquired fifteen (15) fertilized six-day old chicken eggs from the Provincial Breeding office with a permit from the Provincial Veterinarian of Abra, Ruston P. Valera, DVM, served as specimen for the study. The fertilized eggs were further incubated for 10 days right after the acquisition at 37°C. The incubator that was utilized was automatic which means that the position of the eggs during the incubation period was automatically altered by the incubator.

B. Collection and Preparation of Plant Material

The leaves of the oregano *(Origanum vulgare)* were gathered from Poblacion, Peñarrubia, Abra. The leaves were washed thoroughly and immediately preceded for extraction.

C. Aqueous Extract

The materials utilized for the aqueous extraction of the oregano leaves were, juicer, sterilized scissors, Whatman's No.1 filter paper to filter the oregano leaf extract and glass vials to store the extract. The oregano leaves were cut into pieces using a sterilized scissor and placed in a juicer. Afterwards, the aqueous extract was filtered using Whatman's no. 1 filter paper and was placed in glass vials.

D. Chick Chorioallantoic Membrane (CAM) Assay

The materials used for the CAM assay were fertilized chick eggs (6 day-old), egg tray, incubator, parafilm, scalpel and blade.

period to prevent adherence of CAM on the shell membrane. (Acebedo, 2014).

After 10 days of incubation, candling of eggs in a dim room using a flashlight was performed by simply holding the egg in front of the embryo after casting its shadow.

With the use of blade or scalpel, the shell above the embryo was scraped which permitted the cutting of about a 1cm x 1cm window. The shell was carefully removed, mindful of not cutting any portion of the shell membrane. This prevented unwanted substances from entering the chorioallantoic membrane when the shell was removed. For convenience, the egg was placed on an egg tray while making an opening. (Acebedo 2014)

After the peeling of the shell membrane, the chorioallantoic membrane (CAM) was treated with the different dosages of the plant extract using a micropipette for the experimental treatments (100µl concentration, 200µl and 300µl leaf extract for treatments 1, 2 and 3 respectively), the control positive was treated with 100 µl which showed that any substance can still affect the growth of blood vessels and the eggs on the control negative were non-manipulated which served as the standard of normal blood vessel growth. After the CAM was treated, the egg window was subsequently sealed with parafilm. After 4 days of further incubation, the blood vessel formation was quantified, the parafilm was removed and the size of the window to facilitate cutting of the chorioallantoic membrane was enlarged. Approximately 1cm x 1cm CAM was cut and placed on a glass slide, wherein a drop of distilled water was added. A cover slip was placed on top of the CAM and was sealed using nail polish. (Acebedo, 2014)

E. Measuring Blood Vessel Density and Length of Blood Vessels

The materials used for the measurement of blood density and length of the blood vessel were chick chorioallantoic membrane, glass slides, microscope, micrometer and Toupview software.The chick chorioallatoic membrane was observed with the aid of a microscope for the blood vessel count and to determine the number of branching among blood vessels in a random field of view. Using the ToupView software, the length of the blood vessels was measured, a micrometer was used to calibrate the software and the setting was set to 4x magnification. The unit of measurement of the length of the blood vessels was pixels, although it was set to micrometer (µm) in the settings. In the calibration, 100 µm is equal to 400 pixels (note the 4x magnification), the researchers converted the results based on this.

F. Statistical Analysis

The zero values are present; the entire data from trials were transformed by adding 0.5 to the value getting its square root. To determine the significant difference between the different treatments, the gathered data were analyzed through one-way analysis of variance (ANOVA) and was subjected to post hoc test to confirm where the differences occurred between the treatments using the Duncan Multiple Range Test (DMRT).

Results

This section of the project discusses, interprets the results and provides the statistical conditions of the data gathered.

decreased. Growth of veins from the blood vessel is not evident in Treatments 2 and 3 which were treated with 200 µl and 300 µl oregano crude extract, but since zero values were not accepted, the raw data was transformed.

Difference between the Antiangiogenic Activities of the Treatments used on the Chorioallantoic Membrane in terms of Blood Vessel Density

Effects of Origanum vulgare Aqueous Leaf Extract in Inhibiting Angiogenesis on the Chick Chorioallantoic Membrane in terms of Blood Vessel Density

Table 1.1. The mean blood vessel density or the no. of branches from the main blood vessel of the treatments treated with the different amount of Origanum vulgare leaf extract as compared to the control treatment.

TREATMENTS	MEAN OF THE BLOOD VESSEL DENSITY (NO. OF BRANCHES FROM THE MAIN BLOOD VESSEL)
	MEAN
Negative Control: Non-manipulated Egg	7.48
Positive Control: Treated with 100 µl Distilled H_2O	6.36
Treatment 1: 100 µl Extract	1.18
Treatment 2: 200 µl Extract	0.71
Treatment 3: 300 µl Extract	0.71

Table 1.1 shows the mean blood vessel density or the number of branches from the main blood vessel of the treatments treated with the different concentrations of oregano *(Origanum vulgare)* leaf extract as compared to the control treatment. It can be gleaned from the table that negative control or the non-manipulated egg has the greatest number of blood vessel branches with a mean of 7.48. On the other hand, positive control, which is the chicken egg treated with distilled water showed the mean average of 6.36. For treatment 1, the mean average of the blood vessel density is 1.18, while Treatment 2 and 3 treated with 200 µl and 300 µl oregano leaf extract respectively showed no growth of veins from the main blood vessel, but zero values can't be used in experimental design, the raw data was transformed. Hence, the results show 0.71 mean of measurement.

Effects of Origanum vulgare Aqueous Leaf Extract in Inhibiting Angiogenesis on the Chick Chorioallantoic Membrane in terms of Length of Blood Vessels

Table 1.2 displays the length of the main blood vessel (as per branching) of the treatments treated with varying amounts of oregano crude extract. Control negative has a mean length of 4.50 µm. This means that growth of veins from the main blood vessel have occurred every 4.50 µm. Meanwhile, the positive control treated with 100 µl of distilled water has a mean length of 6.36 µm. In the contrary, Treatment 1 treated with 100 µl oregano crude extract has a mean length of 1.48 µm, indicating that the growth of veins from the main the blood vessel

Table 2 displays the f-test or the one-way analysis of variance (ANOVA) of the blood vessel density or the number of branches from the main blood vessel in the chorioallantoic membrane. The result showed the significant differences of the five treatments given with varying amounts of oregano *(Origanum vulgare)* crude extract. It can be gleaned that significance which is at .001 is lesser than the 0.05 level of significance and it is

interpreted as significant, thus, rejecting the null hypothesis. It was evident that there is a significant difference between the blood vessel density among the five treatments. The result reiterated that the oregano *(Origanum vulgare)* leaf extract is effective in inhibiting the growth of blood vessels.

Difference between the Antiangiogenic Activities of the Treatments used on the Chorioallantoic Membrane in terms of Length of Blood Vessels (as per branching)

Table 5 represents the f-test or the one-way analysis of variance (ANOVA) of the length of the blood vessels (as per branching). It showed the significant differences of the five treatments of chorioallantoic membrane given with varying amounts of oregano *(Origanum vulgare)* crude extract.

As presented in the table, the significance which is .000 is lesser than the 0.05 level of significance. It was interpreted as significant and therefore rejects the null hypothesis. It is evident that there is a significant difference between the blood vessel density of the five treatments. The result of the f-test proved that the oregano *(Origanum vulgare)* leaf extract is effective in inhibiting the growth of blood vessels.

Table 1.2. The mean length of main blood vessel (as per branching) of the treatments treated with the different amount of Origanum vulgare leaf extract as compared to the control treatment

TREATMENTS	MEAN OF THE BLOOD LENGTH OF BLOOD VESSELS
	MEAN
Negative Control: Non-manipulated Egg	4.50 µm
Positive Control: Treated with 100 µl Distilled H_2O	6.36 µm
Treatment 1: 100 µl Extract	1.48 µm
Treatment 2: 200 µl Extract	0.71 µm
Treatment 3: 300 µl Extract	0.71 µm

Table 2. The One-Way Analysis of Variance (ANOVA) result of the blood vessel density of the chorioallantoic membrane

BLOOD VESSEL DENSITY						
	Sum of Squares	df	Mean Square	F	Sig.	Decision
Between Groups	134.528	4	33.632	13.020	.001	Rejected
Within Groups	25.831	10	2.583			
Total	160.360	14				

Table 3. The One-Way Analysis of Variance (ANOVA) result of the length of blood vessels (as per branching)

ANOVA						
LENGTH OF BLOOD VESSELS						
	Sum of Squares	df	Mean Square	F	Sig.	Decision
Between Groups	81.456	4	20.364	26.905	.000	Rejected
Within Groups	7.569	10	.757			
Total	89.025	14				

Table 4. Post Hoc Test of the Blood Vessel Density using Duncan Multiple Range Test (DMRT)

BLOOD VESSEL DENSITY				
	TREATMENTS	N	Subset for alpha = 0.05	
			1	2
Duncan[a]	Treatment 2	3	.7100	
	Treatment 3	3	.7100	
	Treatment 1	3	1.1800	
	Control Positive	3		6.3667
	Control Negative	3		7.4867
	Sig.		.740	.413
Means for groups in homogeneous subsets are displayed.				
a. Uses Harmonic Mean Sample Size = 3.000.				

The DMRT result shows that there is a significant difference among the treatments, which supports and confirms the result of the ANOVA conducted on the data gathered from the mean blood vessel density of the chick chorioallantoic membrane from the different treatments.

Similar findings were also observed by Salas and Totaan 2015 on *Selected Philippine Herbal plant extracts as Angiogenesis inhibitors using Chick Chorioallantoic Membrane (CAM) assay*, they observed the antiangiogenic activity of sabungai (*Gynura nepalensis DC*), pandan (*Pandanus odoratissimus L.*) and tsaang-gubat (*Carmona retusa (Vahl.) Masam.*) individually compared with the control treatment. Statistical analysis on the data gathered showed that Sabungai extract was found significantly different to the positive control while no significant difference is observed on the mean number of the branching points of the eggs treated with pandan and tsaang-gubat extracts when individually compared to the positive control.

Table 5. Post Hoc Test of the Length of Blood Vessels using Duncan Multiple Range Test (DMRT).

LENGTH OF BLOOD VESSELS					
	TREATMENTS	N	Subset for alpha = 0.05		
			1	2	3
Duncan[a]	Treatment 2	3	.7100		
	Treatment 3	3	.7100		
	Treatment 1	3	1.4867		
	Control Negative	3		4.4967	
	Control Positive	3			6.5167
	Sig.		.321	1.000	1.000
Means for groups in homogeneous subsets are displayed.					
a. Uses Harmonic Mean Sample Size = 3.000.					

The DMRT result shows that there is a significant difference among the treatments which verifies the result of the ANOVA on the mean length of blood vessels of the different treatments.

Similar results can also be observed from the study conducted by Kalimuthu, Prabakaran and Saraswathy 2014 on the *Antiangiogenic activity of Boucerosia diffusa and Boucerosia truncato-coronata extracts in chick Chorioallantoic Membrane (CAM),* results showed that the cloroform and ethanolic extract of *B.diffusa* showed higher angiogenic activities at 45.7 and 44.4 respectively and showed higher inhibition of vessel number, on the other hand, *B. truncato-coronata* has a higher angiogenic activity at 65.90 and less vessel number was observed in its methanolic extract. Duncan Multiple Range Test (DMRT) on the data gathered showed that there is a significant difference between the antiangiogenic activity of the ethanolic, methanolic, ethyl acetate and chloroform extracts of B.diffusa. It also shows that there is a significant difference between the ethanolic, methanolic, ethyl acetate and chloroform extracts of *B. truncato-coronata.*

DISCUSSION

The results showed that in the application of different quantities of oregano crude extract: treatments 1, 2 and 3, have successfully inhibited the diverging abilities of blood vessels from the main branch and or the growth of blood vessels as depicted from treatments 2 and 3.

This is similar to the results gathered by Jadhav, Mane and Kanase 2011 on the *Antiangiogenic properties of Boerhaavia diffusa extracts in chick Chorioallantoic Membrane (CAM)*, where the acetone-extract showed highest inhibitory activity in angiogenic response in terms of number and area of secondary and tertiary blood vessels; followed by benzene and alcohol extracts.

The National Cancer Institute, 2018 mentioned that angiogenesis inhibitors interfere with blood vessel growth, thus preventing the growth of cancerous tumors. This study supports the significance of the result shown *Origanum vulgare* contains a high percentage of Carvacrol which is at 46.5, followed by γ-terpinene with 13.91 and ρ-cymene with 13.54 (Moradi, et al. 2014).

Similar components were found in the plant utilized by Rick et al., 2017 on their research study entitled *Phytochemical screening, Antioxidant and Antiangiogenic Properties of Oncoba welwitschii (Oliv.) Gilgn and Tetrorchidium oppositifolium(Pax. and Khoffm.), Medicinal Plants from Gabon.* It was mentioned in the study that phenolic compounds, flavonoids and tannins on the plants, which also found in oregano leaf extract, may have preventive effects on chronic diseases such as cancer and heart diseases.

in table 1.1, since the varying amounts of oregano crude extract had decreased and in the case of treatments 2 and 3, completely stopped the growth of blood vessels in the chorioallantoic membrane.

The result on the mean length of blood vessels show that the oregano leaf extract is effective in inhibiting the vascularization of the chorioallantoic membrane, based on table 1.2, as the dosage of oregano crude extract increases, the length of the growth of secondary blood vessels from the main blood vessels also increases, but since there is no blood vessel growth in treatments 2 and 3, measuring of blood vessel was not needed.

It is very evident that the leaf extract shows promising results as a potential alternative to current cancer medications by effectively inhibiting blood vessel formation.

Angiogenesis inhibitors affect the blood vessel growth that helps in the growth of cancerous tumors (National Cancer Institute, 2018). This study supports the significance of the results shown in the table, since the varying amounts of oregano crude extract reduced the growth of blood vessels or the absence of blood vessels grown in the chorioallantoic membrane have been observed.

In connection with the findings of this study, it can be said that the antiangiogenic effects of oregano crude extract found effective through its polyphenolic components, such as flavonoids, anthocyanins and saponins. These results are supported by the article written by Robertson, 2018 that flavonoids aside from being antioxidants, also have anti-viral, anti-cancer, anti-inflammatory and anti-allergic properties. Moreover, anthocyanins play an important role in lowering the risk of cardiovascular disease, cognitive decline and cancer while saponins have an antioxidant effect to cancer cells (Webb, 2014).

Other studies have shown that essential oil components of the oregano plant could also have a contribution to its angiogenesis inhibiting capabilities. The essential oil of the

According to the study conducted by Han, X. and Parker, T. on the anti-inflammatory, tissue remodeling, immunomodulatory, and anticancer activities of oregano (*Origanum vulgare*) essential oil in a human skin disease mode, the component carvacrol is widely used for its antioxidant, antibacterial, antifungal, anticancer, and anti-inflammatory properties.

CONCLUSIONS

Related studies proved that crude leaf extract of *Origanum vulgare* consists of phenolic or polyphenolic substances such as anthocyanins, flavonoids, and saponins, (Bendifallah, et al. 2015). In this study, *Origanum vulgare* crude extract significantly reduces the

length and density of the blood vessels of chick chorioallantoic membrane, indicating its angiogenesis inhibiting capabilities. These results suggest that antiangiogenic effects of crude leaf extract of *Oreganum vulgare* may be mediated via its antioxidant and anticancer effects.

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