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A STUDY ON THE DETERMINATION OF ANTI-ANGIOGENESIS POTENTIAL OF XEROCHRYSUM BRACTEATUM (EVERLASTING FLOWER) ETHANOLIC CRUDE EXTRACT BY CHORIOALLANTOIC MEMBRANE ASSAY OF FERTILIZED CHICK EGGS.

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Abstract

This research study is about the anti-angiogenesis potential of *Xerochrysumbracteatum* (Everlasting) flower ethanolic crude extract. Chorioallantoic membrane assay was utilized, wherein test solutions were introduced directly to the eggs' membrane via a small opening of the egg shell.

One trial was made for the experiment, using a total of 18 chicken eggs, 6 eggs per group. The eggs were incubated for 10 days. In the experiment, the experimental group was treated with the everlasting ethanolic crude extract (EECE), positive control group - Vitamin A and the negative control group was left untreated. The eggs were re-incubated for 2 days to facilitate the absorption of the drug for the analysis. After re-incubation, the eggs were totally opened and separated to the membrane carefully to avoid blood vessels rupture. AngioTool Software was also used for quantitative assessment of various vessel morphometric and spatial parameters including vessel length and density. The data gathered was determined using One-Way ANOVA and Tukey post-hoc test. The P value which is <0.01 level is considered significant. The results showed that Everlasting Ethanolic Crude Extract (EECE) has a significant difference (number of blood vessels) and comparable effects (blood vessels length and density) with the positive control group (Retinoic Acid) for its anti-angiogenesis activity. Basing on its potency and effect, it is therefore affirmative for anti-angiogenesis.

The recommendations of this study are to conduct a study regarding the components of the Everlasting Flower that is responsible of its antiangiogenic property and also conduct a study regarding the determination of the minimal concentration for its antiangiogenic property. Most importantly, to conduct a study separating the antiangiogenic components and more.

Keywords

Antiangiogenic, Chorioallantoic membrane assay, Incubation, Blood vessels, Ethanolic crude extract.

Introduction

Statement of the Problem

The main aim of the study was to determine the anti-angiogenesis activity of the ethanolic crude extract of Everlasting flowers using Chorioallantoic membrane assay on fertilized 8-day old Chicken Eggs specifically through blood vessel growth inhibition expressed in the average number of vessel collaterals.

Specifically, the author seek to answer the following:

1.Determine the number of blood vessels after 48 hours of administrationof the ethanoliccrude extract in different groups;

1.1 Experimental group of Everlasting Flower ethanolic crude extract
1.2 Positive Control group of Retinoic Acid
1.3 Negative Control group (untreated)

2. Determine the percent inhibition of vessel number;

2.1 Experimental group ofEverlastingFlower ethanolic crude extract
2.2 Positive Control group ofRetinoic Acid
2.3 Negative Control group (untreated)

3. Determine the blood vessel length;

3.1 Experimental group ofEverlastingFlower ethanolic crude extract
3.2 Positive Control group of Retinoic Acid

3.3 Negative Control group (untreated)

4. Determine the blood vessel tubule density;

4.1 Experimental group ofEverlastingFlower ethanolic crude extract

4.2 Positive Control group ofRetinoic Acid

4.3 Negative Control group (untreated)

5.Determine the significant difference in the mean vessel length, density and percent inhibition of vessel number between experimental and control group.

Review of literature

Review of literature Cancer is one of the severe human diseases which cause increasing mortality every year in the world. Tumor growth and systemic metastasis are highly dependent on angiogenesis,(1). It is a process involved in growth of new capillaries from existing ones, which is a regular process that rarely occurs under normal conditions. Many diseases are driven by persistent unregulated angiogenesis,(2).

Angiogenesis is a normal process in growth and development of blood vessels (1), and is a process essential for tumor growth. It is a tightly regulated process that involves signaling and extracellular matrix that induces the migration of endothelial cells to target the areas which are sources of proangiogenic signaling compounds (3). Thus inhibition of angiogenesis is the prime target to afflict the growth of solid tumors. Angiogenesis promotes growth of tumors by providing nutrients and oxygen, meanwhile facilitating tumor invasion and metastasis (3),hence a target for cancer chemotherapy.

Antiangiogenic therapy aims to prevent the formation of new vessels around tumors, which remains in the dormant state until it can stimulate blood vessel growth from nearby pre-existing capillaries (4). Thus the antiangiogenic therapy results in ablation of tumor vessels and prevents cancer which is more advantageous and is less toxic than chemotherapy agents (5). Thus identifying antiangiogenic pathway and synthesis of novel drug for therapeutic purpose are underway and natural products still represent an important source of leads for drug development. Therefore, there is a need to study angiogenesis in greater detail and in the relevant disease and tissue to allow the development of new/improved therapeutic strategies.

Helichrysum bracteatum known today as Xerochrysum bracteatum (Everlasting) is a stout annual herb growing to a height of 30 to 60 centimeters, with terete and sparingly branched stems. Leaves are alternate, oblong-lanceolate, with entire margins, and narrowed at the base. Blade is green on both sides. Flower head is terminal, up to 6 centimeters across, golden yellow, pink, orange to ivory white, enclosed by strawlike imbricated bracts of varying colors of red, yellow, brown and white.(6)

FIGURE 1. Everlasting Flower

Everlastings were used by the Egyptians to decorate the statues of their gods. Greeks used Everlastings mixed with honey to soothe burns. In the Victorian era, Everlastings were used to make fireplace screens. They were also used instead of houseplants to decorate their living rooms (7).

Helichrysum is a natural medicinal plant that's used to make a beneficial essential oil that boasts many different full-body benefits due to its anti-inflammatory and **antioxidant** properties. Helichrysum essential oil, in the form of *Helichrysum italicum* extract, has been established in various experimental studies to have strong abilities to lower **inflammation** due to several mechanisms: inflammatory enzyme inhibition, **free radical** scavenging activity and corticoid-like effects, (8).

Phytochemical studies conducted by (9) have revealed that the plant is rich in flavonoids and other water soluble polyphenolic compounds. The presence of monoterpenes such as α-pinene, 1,8-cineole and *p*-cymene were found. Its profile consisted of a variety of sesquiterpenes in low concentrations with β-caryophyllene dominating. Chemical constituents are responsible for its many activities and is a big basis for its antioxidant properties.

The main goal of this research was to determine the anti-angiogenic activity of Everlasting on the chorioallantoic membrane of 8-day old fertilized chicken eggs.

Exposure and utilization of the easily assesible, affordable plants found in Philippines as a cheaper medicine formulation for anti-cancer.

Materials and Methods

This research utilized the experimental trial which determined the anti-angiogenesis effect of ethanolic crude extractof *Xerochrysum bracteatum* (Everlasting) flowers on Chrorioallantoic membrane of an 8-day old fertilized Chicken Eggs.The plant samples were collected at Casay Dalaguete, Cebu. The test subjects were procured respectively.

The study was conducted in the Laboratory Room of the College of Pharmacy. The eggs were incubated and treated in an incubator found in the Instrumentation Room of the College of Pharmacy. Incubation of the eggs and experimentation was conducted on a hatchery.

Eighteen 10-day old fertilized chicken eggs were used as test subjects. Each group namely; positive control group, experimental group, and negative control group were randomly assigned with six eggs per group.

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The researcher utilized the AngioTool Software and Canon DSLR D-1100 that determined the angiogenesis. The AngioTool Software provided a semi-automated analysis of angiogenesis by measuring tubule density or vessel number, length and number of tubule junctions. Record sheets were utilized for recording manual data during the experiment.

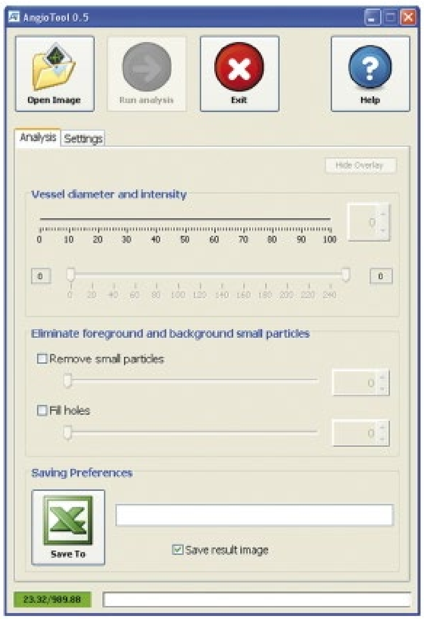

FIGURE 2. AngioTool Software

Results and Discussion

TABLE 1. Percent Inhibition of Blood Vessels

Percent Inhibition of Number of Blood Vessels			
Trial	Positive Control	Negative Control	Experimental group
1	41.02564103	0	74.35897436
2	38.88888889	0	83.33333333

3	52.5	0	72.5
4	48.71794872	0	66.66666667
5	50	0	53.125
6	44.73684211	0	81.57894737
MEAN	45.98214286	0	72.32142857

The results show that there is a mean blood vessels percent inhibition of 72.32% for the Experimental Group, 45.98% for the Positive Control while the negative control exhibited 0%.

ANALYSIS AND DISCUSSION OF FINDINGS

TABLE 1A. Analysis of Variance of Blood Vessels

Analysis of Variance

Source of Variation	*SS*	*Df*	*MS*	*F*	*p-value*	*F crit*
Between Groups	18,575.3384	2	9,287.6692	221.9465	0.0000	3.5546
Total	19,328.5742	20				

The results of the ANOVA yielded a p-value < 0.01, therefore the null hypothesis is rejected which means that there is a significant difference between the percent (%) inhibition of blood vessels of the experimental group and the control groups in the experiment. Due to the p-value resulting to < 0.01, a post-hoc analysis was done to determine which sample is considered significantly different from the other.

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TABLE 1B. Post Hoc Analysis of Blood Vessels

Post Hoc Analysis :Tukey HSD Test for Differences Between Means - Number of Blood Vessels

Groups	*p-value*	*Interpretation*
Positive Control v Negative Control	0.00032	Significant
Positive Control v Experimental group	0.000027	Significant
Negative Control v Experimental group	0.0000354	Significant

According to the post-hoc analysis on the results, there's a significant difference between the all groups with a p value of 0.00032, 0.000027, and 0.0000354, respectively.

The positive control group compared to the negative control group yielded the result of p-value 0.00032 which is expectedly significant thus there is inhibition of blood vessels.

The positive control group compared to the experimental group yielded the result of p-value 0.000027 which is significant thus the potency of the effect of the treatment on the experimental group is greater than that of the positive control group.

The negative control group compared to the experimental group yielded the result of p-value 0.0000354 which is significant thus that there is still inhibition of the number of blood vessels.

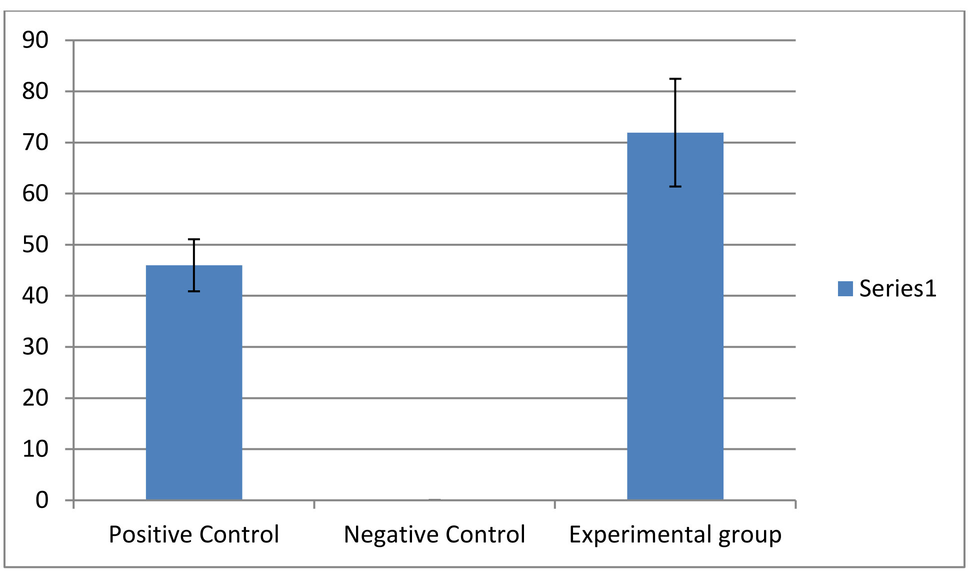

FIGURE . Post Hoc Analysis of Blood Vessels

The bar graph shows that the standard deviation between groups of experimental and positive control did not intersect and thus doesn't have the same potency or effect. The experimental group has higher anti-angiogenic effect compared to the positive control group with regards to the blood vessel number.

TABLE 2. Percent Inhibition of Blood Vessel Length

Percent Inhibition of Blood Vessel Length			
Trial	Positive Control	Negative Control	Experimental group
1	47.29876249	0	36.60107822
2	65.27379316	0	81.94101359
3	91.28833446	0	94.42953022
4	34.35789581	0	83.60397405
5	58.74960975	0	77.20086479
6	51.23016358	0	73.3106029
MEAN	70.71579865	0	83.75663805

The results show that there is a mean blood vessel length percent inhibition of 83.76% for the Experimental Group, 70.72% for the Positive Control while the negative control exhibited 0%.

TABLE 2A. Analysis of Variance of Blood Vessel Length

Analysis of Variance

Source of Variation	*SS*	*df*	*MS*	*F*	*p-value*	*F crit*
Between Groups	21,447.7418	2	10,723.8709	49.9903	4.4788E-8	3.5546
Total	25,309.0882	20				

The results of the ANOVA yielded a p-value < 0.01, therefore the null hypothesis is still rejected which means that there is a significant difference between the percent (%) inhibition of blood vessel length of the experimental group and the control groups in the experiment. Due to the p-value resulting to < 0.01, a post-hoc analysis was done to determine which sample is considered significantly different from the other.

TABLE 2B. Post Hoc Analysis of Blood Vessel Length

Post Hoc Analysis :Tukey HSD Test for Differences Between Means - Vessel Length

Groups	*p-value*	*Interpretation*
Positive Control v Negative Control	0.000313	Significant
Positive Control v Experimental group	0.0322	Insignificant
Negative Control v Experimental group	0.000881	Significant

According to the post-hoc analysis on the results, there's a significant difference between the positive conrol group and negative control group with the p-value 0.000313; also negative control group and experimental group with the p-value 0.000881. The positive control group and experimental group yielded the p-value 0.0322 which is insignificant.

The positive control group compared to the negative control group yielded the result of p value 0.000313 which is expectedly significant thus there is inhibition of blood vessels length.

The positive control group compared to the experimental group yielded the result of p-value 0.0322 which is insignificant thus the potency of the effect of the treatment on the experimental group is the same with that of the positive control group on the blood vessels length.

The negative control group compared to the experimental group yielded the result of p-value 0.000881 which is significant thus that there is still inhibition of blood vessels length.

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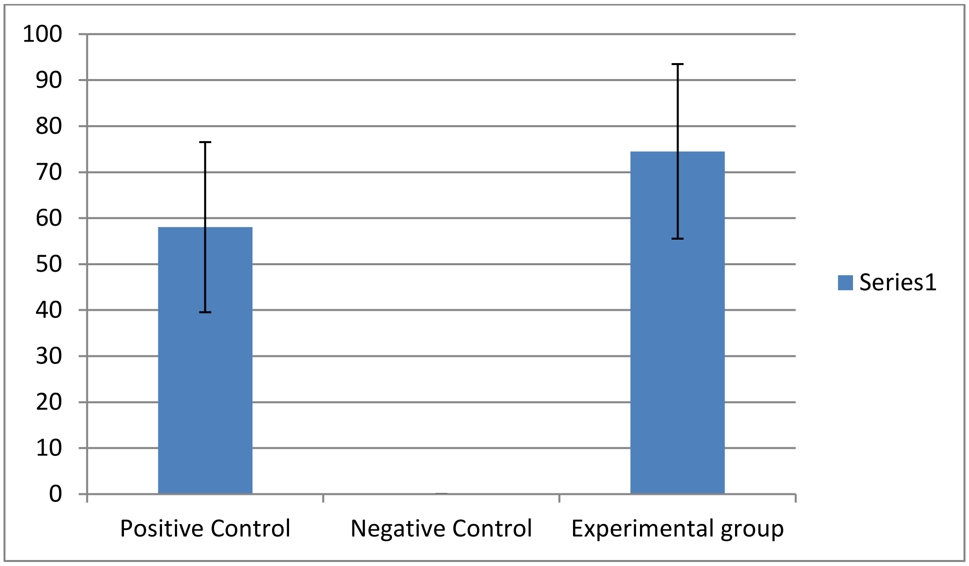

FIGURE . Post Hoc Analysis of Blood Vessel Length

The bar graph shows that the standard deviation between groups of experimental and positive control intersected. Thus, experimental group has the same anti-angiogenic effect with the positive control group with regards to their blood vessel length inhibition.

TABLE 3. Percent Inhibition of Blood Vessel Density

Percent Inhibition of Blood Vessel Density			
Trial	Positive Control	Negative Control	Experimental group
1	97.70232025	0	97.80949502
2	99.88242687	0	97.44066233
=+*	99.37146657	0	99.51009843
4	99.05577032	0	99.55236148
5	97.31478592	0	98.77249557
6	96.83169034	0	99.68340941
MEAN	98.40878086	0	98.83580453

The results show that there is a mean blood vessel length percent inhibition of 98.41% for the Experimental Group, 98.84% for the Positive Control while the negative control exhibited 0%.

TABLE 3A. Analysis of Variance of Blood Vessel Density

Analysis of Variance

Source of Variation	*SS*	*df*	*MS*	*F*	*p-value*	*F crit*
Between Groups	45,348.8740	2	22,674.4370	33,005.3237	0.0005	3.5546
Total	45,361.2399	20				

The results of the ANOVA yielded a p-value < 0.01, therefore the null hypothesis is still rejected which means that there is a significant difference between the percent (%) inhibition of blood vessel density of the experimental group and the control groups in the experiment. Due to the p-value resulting to < 0.01, a post-hoc analysis was done to determine which sample is considered significantly different from the other.

TABLE 3B. Post Hoc Analysis of Blood Vessel Density

Post Hoc Analysis :Tukey HSD Test for Differences Between Means - Vessel Density

Groups	*p-value*	*Interpretation*
Positive Control v Negative Control	0.000983	Significant
Positive Control v Experimental group	0.0219	Insignificant
Negative Control v Experimental group	0.005	Significant

According to the post-hoc analysis on the results, there's a significant difference between the positive conrol group and negative control group with the p-value 0.000983; also negative control group and experimental group with the p-value 0.005. The positive control group and experimental group yielded the p-value 0.0219 which is insignificant.

The positive control group compared to the negative control group yielded the result of p value 0.000983 which is expectedly significant thus there is inhibition of blood vessels density.

The positive control group compared to the experimental group yielded the result of p-value 0.0219 which is insignificant thus the potency of the effect of the treatment on the experimental group is the same with that of the positive control group on the blood vessels length.

The negative control group compared to the experimental group yielded the result of p-value 0.005 which is significant thus that there is still inhibition of blood vessels density.

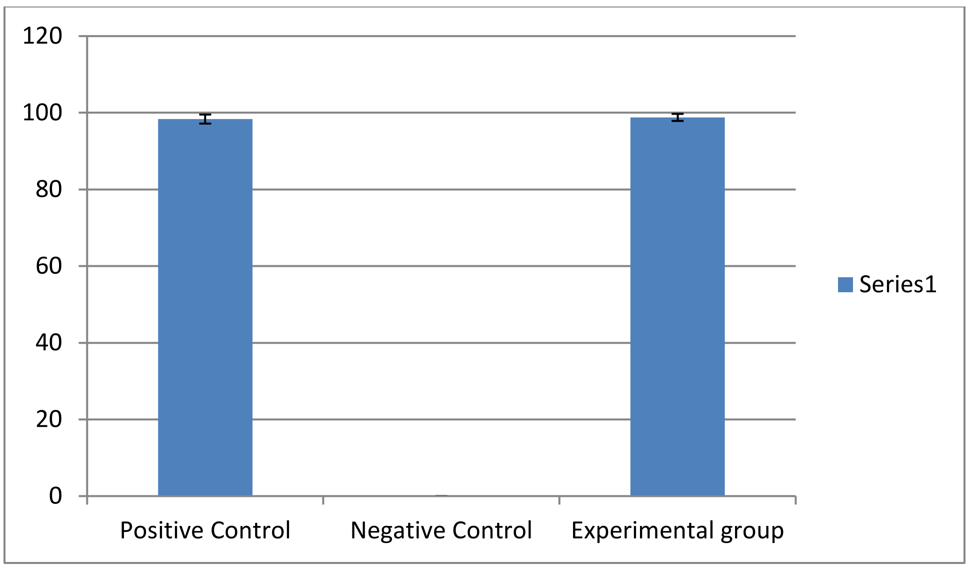

FIGURE . Post Hoc Analysis of Blood Vessel Density

The bar graph shows that the standard deviation between groups of experimental and positive control intersected. Thus, experimental group has the same anti-angiogenic effect with the positive control group with regards to their blood vessel density inhibition.

FINDINGS

Based on the data presented, the Everlasting Ethanolic Crude Extract exhibited the greatest percent (%) inihibition in blood vessel number (72.32%), blood vessel length (83.76%) and blood vessel density (98.84%) compared to the positive control (Retinoic Acid)) which exhibited the percent (%) of inhibition in blood vessel number (45.98%), blood vessel length (58.03%) and blood vessel density (98.34%). Therefore, the Everlasting Ethanolic Crude Extract had greatest effect on the dependent variables and is considered to be effective in inhibiting angiogenesis while on the other hand, the Positive Control has a lesser potency than that of the Everlasting Ethanolic Crude Extract.

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