Angiosuppressive and Teratogenic Influence of an Edible Medicinal herb, Insulin plant (*Costus igneus*) in Mallard Duck (*Anas platyrhynchos*) Embryos.

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Abstract— **It has been found out that medicinal plants are potential sources of anticancer compounds. Thus, this study was conducted to evaluate the anti-angiogenic potential of the crude ethanolic extract from Insulin plant (*Costus igneus*) through chorioallantoic membrane (CAM) vascularity and teratogenicity assays in mallard duck (*Anas platyrhynchos*) embryos. Each of the seven treatments - 5.0%, 1.0%, 0.1%, 0.06% and 0.01% extracts, alpha-tocopherol (positive control) and pure olive oil (negative control) was randomly administered by injection into the air cell of twenty 8-day old embryos that were incubated at 37.5º C. On the 12th day of incubation, five living embryos in each treatment were sacrificed. Using Image J software developed by Wayne Rasband (2010), primary, secondary and tertiary blood vessels were counted, and the diameters (in cm) of primary and secondary blood vessels were measured in a representative 4 by 4 cm fractal segment of the CAM of five alive embryos in each treatment group. On the 18th day of incubation, the remaining ten embryos in each group were examined for gross external morphological abnormalities. Results showed that the 5.0% extract was fatal. The results revealed that there is an insignificant difference among the values of the secondary blood vessels, tertiary blood vessels, and total number of blood vessels, diameter of primary blood vessels and diameter of secondary blood vessels with respect to the positive and negative control. The teratogenicity assay revealed that the results are statistically insignificant for gross morphological abnormalities. Therefore, the results revealed that the extracted crude ethanolic substance of *C. igneus* did not contain components that can significantly inhibit angiogenesis. Furthermore, these results revealed that Insulin plant (*C. igneus*) has no teratogenic effect.**

***Keywords*---Anti-angiogenecity, Chorioallantoic Membrane Vascularity Assay and Teratogenicity Assay.**

I. Introduction

Cancer is a chief cause of diseases worldwide. There is an estimated 14.1 million new cases of cancer in the world in the year 2012; 53% in males and 47% in females. The World Age Standardized (AS) occurrence rate shows that there are 205 new cancer cases for every 100,000 men in the world and 165 for every 100,000 females [32]. In 2008, there were 7.6 million deaths for males and 8.2 million deaths for female in the year 2012 due to cancer, with an increasing rate of 7.89% from the year 2008 to 2012. Deaths due to cancer are projected to continue rising with an estimated number of 13.1 million deaths in 2030 [34].

There are various types of cancer treatment employed in the field of medicine. Among these are chemotherapy, radiation and surgical operations. However, the quantity of deaths due to cancer is still increasing. The current cancer medications are quite expensive; thus, some of the underprivileged sectors have resorted to traditional medicine to cure their diseases [22].

Tumors rely on angiogenesis and the attain food and nutrients in order for them to survive, grow and metastasize [8]. Since tumor growth is desperate for angiogenesis and metastasis, anti-angiogenic treatment is therefore a highly promising therapeutic approach [14].

Herbal medicine made from medicinal plants are used for prevention and treatment of diseases [3]. Several herbs have been studied as having anti-angiogenic properties which are important because it can be used to examine the angiogenesis in cancerous tissue and further inhibit the development such tissues [2].

Costus igneus is a medicinal plant known for its ability to cure diabetes [5]. In southern India, it usually grows as an ornamental plant and its leaves are used as a dietary supplement in the treatment of diabetes mellitus [5]. It can also be found in the Philippines. Aside from being a cure for diabetes, the ethanolic extract of leaves of *C. igneus* was found to have anti-proliferative and anti-cancer potential [5]. However, its ability to exert anti-angeogenicity is not yet studied. Thus, the purpose of this study is to examine the angiogenic and teratogenic effects of the crude ethanolic extract from *C. igneus* in mallard duck (*Anas platyrhynchos*) embryos.

II. Statement of the Problem

The ethanolic extract from the leaves of *C. igneus* has been found to have anti-proliferative and anti-cancer potential in *in-vitro* mammalian fibrosarcoma (HT- 1080) cells [5]. Yet, there has been no tests if this plant has the ability to kill cancer cells through anti-angiogenicity. Its anti-angiogenesis activity is yet to be examined and its effect on the process of development is still unknown.

Hence, this study will specifically answer the following questions:

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1. *Can the crude ethanolic extract from Costus igneus inhibit angiogenesis in Anas platyrhynchos embryos?*
2. *Is the angiosuppressive activity of the crude ethanolic extract of C. igneus concentration- dependent?*
3. *Can the crude ethanolic extract from C. igneus cause teratogenic effects in A. platyrhynchos embryos?*

III. Scope and Limitation of the Study

C. igneus samples were collected from Marawi City. The concentrated ethanolic extract was diluted to different concentrations: 0.01%, 0.06%, 0.1%, 1.0%and 5.0%. The treatments that were used in this study include: olive oil as negative control, alpha- tocopherol as positive control and the five concentrations of plant extracts (0.01%, 0.06%, 0.1%, 1.0% and 5.0%) which were administered on each of the 20 replicates on the 8th day of duck embryo incubation.

Angiosuppressive activity was determined using the chorioallantoic membrane (CAM) assay in mallard duck embryos. After 12 days of incubation, the embryos were examined for the number of formed blood vessels with the use of Image J software version 1.45s [33]. One representative fractal segment of 4 by 4 cm was examined in each embryo. The primary blood vessel nearest to the heart was used as the reference point to get the fractal segment. The primary blood vessel (PBV) is the largest blood vessel; secondary blood vessels (SBV) are the blood vessels that branch out from the primary blood vessel; and the tertiary blood vessels (TBV) are the blood vessels that branch out from secondary blood vessels [28] as cited by [29]. Only the diameter of PBV and SBV were measured using the Image J software version 1.45 s.

Mortality rate was observed 24-96 hours after the treatment and also observed during the 18th day of incubation which is 240 hours after the administration of treatments. Gross morphological characteristics of the treated embryos not used in the CAM assay were observed on the 18th day of incubation period via destructive sampling. The body weights of embryos were also determined using an analytical balance. The body length, eye diameter, the lengths of the beak, neck, forelimbs and hind limbs were measured using a ruler and Image J software. All measurements, except for the body length were divided by the measure of the body length of the examined embryo to remove its influence on those measurements.

The internal organs of the embryos were not histologically examined. Furthermore, the bioactive components of the crude ethanolic extract from *C. igneus* that was responsible for its angiogenesis-inhibition activity and teratogenic effects were not isolated and identified. The specific mechanisms of action of these bioactive components were also not investigated.

The bioactive components of *C. igneus* crude ethanolic extract that are responsible for its angiosuppresive activity were not determined and their specific mechanisms of action were not investigated.

IV. Related Literature

Tumors stimulate angiogenesis through the secretion of various growth factors such as bFGF and VEGF that can induce capillary growth into the tumor, which some researchers suspect supply demand nutrients, allowing for tumor expansion. In 2007, it was discovered that cancerous cells stop from producing the anti-VEGF enzyme PKG.

In normal cells, PKG apparently limits beta-catenin, which craves for angiogenesis. Other clinicians believe angiogenesis really serves as a waste pathway, taking away the biological end products secreted by rapidly dividing cancer cells. In either case, angiogenesis is a necessary and required step for transition from a small harmless cluster of cells, often said to be about the size of the metal ball at the end of a ball-point pen, to a large tumor. Angiogenesis also commands for the spread of tumor, or metastasis. Single cancer cells can break away from an established solid tumor, enter the blood vessel and be carried to a distant site, where they can implant and begin the growth of a secondary tumor. Evidence recommends that the blood vessel in a given solid tumor may, in fact, be mosaic vessels, composed of endothelial cells and tumor cells. This mosaicity allows for substantial dropping of tumor cells into the vasculature, possibly contributing to the appearance of circulating tumor cells in the peripheral blood of patients with malignancies. The subsequent growth of such metastases will also require a supply of nutrients, oxygen and a waste disposal pathway [25].

Cancer, also known as a malignant tumor, is a group of diseases including abnormal cell growth with the potential to attack or spread to other parts of the body. There are over 100 several types of known cancers that can affect humans [25].

For the past 40 years, great attention has been incited for recognition of possible therapeutic principles of controlling angiogenesis. Angiogenic-stimulation can be therapeutic in ischemic heart disease, peripheral arterial disease, and wound healing. Furthermore, angiogenic-inhibition can also be therapeutic for cancer, ophthalmic conditions, rheumatoid arthritis, and other diseases [1].

A suggestion is made that combining the use of standard cytotoxic chemotherapies and angiogenesis inhibitors may produce complementary therapeutic benefits in the treatment of cancer [4]

Chorioallantoic Membrane (CAM) assay

Chorioallantoic Membrane (CAM) assay is widely employed as an *in vivo* system to study antiangiogenesis [17]. Aside from the CAM assay, there are also other classical assays for studying angiogenesis *in vivo* such as the hamster cheek pouch, rabbit ear chamber, dorsal skin, air sac and the iris and a vascular cornea of the rodent eye [13]. In spite of the availability of these other tests, the CAM assay is favored greatly because it offers the advantage of being relatively inexpensive and lends itself to large-scale screening, with the use of various antiangiogenic substances that examine their effectiveness [12]. CAM is an extraembryonic membrane that serves as gas exchange surface and is supported by vast capillary network. It is formed via the fusion of the chorion and the allantois. It is the mesodermal layer of the allantois that fuses with the adjacent mesodermal layer of the chorion thereby creating a double layer of mesoderm: the chorionic component which is somatic mesoderm and the allantoic component which is splanchnic mesoderm. In this double layer, an extremely rich vascular network develops which is connected to embryonic circulation by the allantoic arteries and veins [13]. Due to this extensive vascularization and ease of access, CAM models have long been

used in studying morphofunctional aspects of angiogenesis process *in vivo* and in studying the effectiveness and mechanism of action of pro- and anti-angiogenic substances from samples of plant extracts [12]; [20].

A. platyrhynchos Teratogenicity Assay

Congenital malformation is an anatomical or structural abnormality present at birth. These malformations may be caused by genetic factors or environmental insults or a combination of the two that occur during prenatal development [22]. Traditionally, the avian embryo is an excellent model for the study of development, as eggs are easy to obtain and manipulate [25]. Like mammals, avian embryos have an allantois and their developmental pathways are highly conserved, thus avian models have biomedical relevance. One feature of avian as a model is the fact that fertile eggs are inexpensive and the embryo develops rapidly, allowing for greater outputs. Secondly, the absence of a placenta allows the direct study of an agent's embryotoxic effects [16]. Furthermore, toxicological and gross morphological structure research on birds may be directly relevant to human health for mechanistic and descriptive studies and indirectly relevant as monitors of the environment [6].

V. METHODOLOGY

Embryo from avian eggs is a well-known model for developmental biology studies has also proven its effectiveness for toxicology researches. Fertile eggs are affordable and the embryo develops rapidly which saves time and allows high output [16]. In this study, duck eggs (*Anas platyrhynchos*) were used as the animal test system. The chorioallantoic membrane (CAM) vascularity assay which is based on [19], with some adjustments based on Wang [20], Liu [7], Muslim [8], Tantiado [18], Chan [28] and Raga [11] was employed to evaluate the angiogenesis- stimulation or inhibition property of the test extract. It is a standard test in assessing the angiogenesis inhibiting or stimulating properties of several compounds. It is also probably the most widely used *in vivo* assay for studying angiogenesis. The advantages of CAM assay include its affordability, reliability, simplicity and suitability to large-scale screening which are important criteria in choosing a method [13].

The duck embryos were also utilized as a teratogenicity test system in this study which helped examine the toxicity of the extract. The avian embryo has been an established test system for evaluating teratogenic effects during embryonic development [29].

Preparation of Treatments

The experimental plants were collected in Marawi City, Lanao del Sur. The plant's identity was identified first using The Flora of Manila [27]. It was then confirmed by Professor Fatimah M. Natangcop, botanist in the Biology Department of Mindanao State University, Marawi City. Only the leaves of the *C. igneus* were used in this study. Which were washed thoroughly and then cut into tiny pieces using a pair scissors. The leaves were placed in a large net at room temperature (24° C) for two weeks. After drying, the dried leaves were stored in a transparent container for future use.

The dried leaves were ground using an electric grinder and then ethanolic extraction was done based on the methodology illustrated by Guevara [24] as cited by Goling [29].

Ninety-five percent (95%) ethanol was used as the extracting medium. About 3,800 ml of absolute ethanol was diluted with distilled water to a total volume of 4,000 ml. Then, about 500 grams of the ground plant material was weighed in a beaker using an electronic weighing scale. It was then soaked in the prepared ethanol solution in an Erlenmeyer flask for 48 hours. The flask was covered with foil to prevent contamination. Subsequently, the soaked material was filtered through a glass funnel using a whattman-1 filter paper. After filtration, the flask and the plant material were rinsed with a small portion of 95% ethanol to obtain the remaining extract.

The crude ethanolic extract was concentrated to a volume of 75 ml in a rotary evaporator at the research room of the Biology Department, College of Natural Sciences and Mathematics of the Mindanao State University, Marawi. This was done at a temperature of 40 degrees Celsius until the extract is almost completely dried. The extract was carefully removed from the flask and transferred to another Erlenmeyer flask. In order to remove excess water from the concentrated extract, it was transferred to sterile petri plates and heated in an oven with a controlled temperature of 40-45 degrees Celsius. The plant extract was properly labeled and then stored at temperatures between 10-15 degrees Celsius inside a refrigerator.

The concentrated extract was diluted to 0.01%, 0.06%, 0.1%, 1.0% and 5.0% concentrations with pure olive oil as the solvent. The extract should partially be dissolved in the solvent. With the use of the pipette, it helped the extract to attain the different concentration needed for the study. Ten (10 ml) of each extract concentration was prepared with the aid of beakers, graduated cylinder and syringes. These were stored in labelled test tubes covered with foil in a test tube rack. Pure olive oil and alpha-tocopherol [21],[26] were used as the negative and positive controls, respectively. The pure olive oil used was Dona Elena pure olive oil manufactured by Fly Ace Corporation from 607 Elcano Street, Binondo, Manila, whereas the alpha-tocopherol used was Myra-e (400 I.U.) capsule manufactured by PT Darya-Varia Laboratoria Tbk, Gunung Putri, Bogor, Indonesia.

Collection of Experimental Eggs

One-day old duck embryos were utilized in the study. A total number of 140 duck eggs were obtained from a licensed commercial supplier in Pala-o, Iligan City. For each of the six treatments, there were 20 replicates in each seven treatments. Seventy (70) of 140 duck eggs were used for CAM assay which were incubated up to 12th day and the other 70 were used for teratogenicity test which were incubated up to 18th day. To prevent bacterial contamination, 70% ethanol was used for cleaning the eggs with the aid of cotton swab.

Chorioallantoic Membrane Vascularity Assay

One-day old fertilized duck eggs were incubated. This was done at a temperature of 37.5 degrees Celsius and at constant humidity of approximately 80%. To examine the viability of

the embryos prior to treatment, the eggs were individually candled at 8th day with the aid of candler lamp. To determine if the eggs contain living embryos, the blood vessels and the heartbeat are the most useful features. Blood ring was observed on non-viable embryos.

The portion of the egg that was injected on the 8th day was disinfected by wiping it with 70% ethanol using a cotton. Also, using an 18-gauge size tuberculin needle, a small tiny hole enough for the syringe to enter was carefully made at the blunt end (air space) of the egg. A volume of 0.2 ml of each treatment was injected in the air cell of the embryo 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. Treatments were administered using aseptic technique to prevent contaminations. In order to dispense each treatment, a different syringe was used in every concentration. The opening was carefully sealed with melted paraffin with an aid of a spatula after inoculations. The paraffin was melted in an evaporating dish over an alcohol lamp. The eggs were then returned to the incubator until day 12. The viability of the eggs was monitored through examining whether they have become rotten. Dead embryos were recorded at 24, 48, 96, 240 hr. (18th day) after the treatment administration. Mortality rates were calculated using the formula:

$$\text{Mortality Rate} = \frac{\text{Number of dead individuals}}{\text{total number of sample preparation per group}} \times 100$$

Blood vessels counting

On the 12th day of incubation, the remaining eggs were placed on their lateral sides to position the CAM and the embryo. Ten (10) out of 20 replicates were randomly selected for CAM examination. The paraffin seal was first removed then the CAMs were harvested by removing the hard shell, leaving intact the soft membrane covering the embryo. These were carefully placed in a sterilized disposable petri dish with the aid of a spatula. The CAMs were then properly spread for better examination. The CAMs were photographed using a 14.1 megapixels Nikon digital camera which were examined using the Image J software, version 1.45s. For the counting of the primary, secondary and tertiary blood vessels and the measurements of primary and secondary blood vessels in diameter, a representative fractal segment of 4 by 4 cm in each CAM was examined. The primary blood vessel nearest to the heart served as the reference point in obtaining the fractal segment. The largest blood vessel from the heart was designated as the primary blood vessel (PBV); the secondary blood vessels (SBV) were those that branched out from the primary blood vessel; and the tertiary blood vessels (TBV) are those that branched out from the secondary blood vessels [28] as cited by [29] Percent vascularity inhibition was calculated using the equation [20]:

$$\frac{\textit{Vessel number in CAM treated by negative control} - \textit{Vessel number in CAM treated by plant extract}}{\textit{Vessel number in CAM treated by negative control}} \times 100$$

Teratogenicity Assay

The remaining alive and treated embryos were examined on the 18th day. Embryos were examined if there were gross morphological anomalies that have occurred. The control embryos were compared to these embryos and abnormalities were recorded. The body weight and body length were recorded to calculate the BMI. Body weight was determined using an analytical balance while the body length was measured using the Image J software version 1.45s. BMI was calculated using the formula:

$$\text{Body mass index (BMI)} = \frac{\text{Body weight in milligrams}}{(\text{body length in mm})^2}$$

Gross morphological effects were measured in terms of the eye diameter and the lengths of the beak, neck, forelimbs and hind limbs. To obtain the eye diameter, the mean of the right and left eye diameter was used. Forelimb and hind limb lengths were obtained by getting the mean of the right and left limb measurements. To remove the influence of body length on those measurements, all measurements are divided by the body length of the examined embryo.

Photography

The photographs of the CAMs and the embryos were taken using a 14.1 megapixels Nikon digital camera. The photographs were then analyzed using the Image J version 1.45s, open access software.

Experimental Design and Statistical Analysis

On the 8th day of incubation, the six treatments namely (+) Positive Control, (-) Negative Control, (0.01) 0.01%, (0.06) 0.06%, (0.1) 0.1%, (1) 1%, (5) 5% were randomly assigned to eggs using the fishbowl technique. The type of the treatment and the replicate number was marked on the eggs with a labeling tape. This was repeated without replacement until all eggs have been assigned with treatments.

The following data were expressed as mean ± standard error. These were analyzed by Kruskal-Wallis test and one way analysis of variance (ANOVA).

P values less than or equal to 0.05 was considered as indicative of significance. The statistical tests were conducted using the Statistical Package for the Social Sciences (SPSS) Version 14.0 and Microsoft Excel 2010.

1) *Primary blood vessel (PBV)*
2) *Secondary blood vessel (SBV)*
3) *Tertiary blood vessel (TBV)*
4) *Total number of blood vessel (PBV + SBV + TBV)*
5) *Primary blood vessel thickness*
6) *Secondary blood vessel thickness*
7) *Body weight*
8) *Body length*
9) *Body mass index (BMI)*
10) *Eye diameter*
11) *Beak length*
12) *Neck length*
13) *Forelimb length*
14) *Hind limb length*

VI. FINDINGS

A. CHORIOALLANTOIC MEMBRANE VASCULARITY ASSAY

Table 1. Number of secondary blood vessels per 4 x 4 cm representative fractal segment of chorioallantoic membrane of duck embryos treated with the test extract

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	2	2	2	2	2	10.00	2.000
0.06% extract	2	2	2	2	2	10.00	2.000
0.1% extract	2	2	2	3	2	11.00	2.200
1.0% extract	2	2	2	2	2	10.00	2.000
5.0% extract	2	2	2	2	2	10.00	2.000
Alpha-tocopherol	2	2	2	2	2	10.00	2.000
Pure Olive Oil	2	2	2	2	2	10.00	2.000

The mean values of the number of secondary blood vessel analyzed using the Kruskal-Wallis has revealed that there is no significant difference among the different treatments (P = 0.423)

Table 2. Number of tertiary blood vessels per 4 x 4 cm representative fractal segment of chorioallantoic membrane of duck embryos treated with the test extract

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	32	10	6	7	8	63.00	12.600
0.06% extract	8	6	14	17	5	50.00	10.000
0.1% extract	8	17	6	21	5	57.00	11.400
1.0% extract	15	8	19	8	12	62.00	12.400
5.0% extract	20	5	10	12	10	57.00	11.400
Alpha-tocopherol	8	10	19	13	11	61.00	12.200
Pure Olive Oil	8	16	7	27	20	78.00	15.600

The Kruskal-Wallis test revealed that there is no significant difference among the different treatments (P = 0.858)

Table 3. Total number of blood vessels per 4 x 4 cm representative fractal segment of chorioallantoic membrane of duck embryos treated with the test extract

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	35	13	9	10	11	78.00	15.600
0.06% extract	11	9	17	20	8	65.00	13.000
0.1% extract	11	20	9	25	8	73.00	14.600
1.0% extract	18	11	22	11	15	77.00	15.400
5.0% extract	23	8	13	15	13	72.00	14.400
Alpha-tocopherol	11	13	22	16	14	76.00	15.200
Pure Olive Oil	11	19	10	30	23	93.00	18.600

The result of Kruskal-Wallis test revealed that there is no significant difference among the different treatments (P = 0.858)

Table 4. Diameter (in mm) of primary blood vessels per 4 x 4 cm representative fractal segment of chorioallantoic membrane of duck embryos treated with the test extract

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.43	0.46	0.46	1.17	0.74	3.25	0.65
0.06% extract	0.75	0.69	0.65	0.74	0.45	3.29	0.66
0.1% extract	0.78	0.42	0.89	0.75	0.79	3.63	0.73
1.0% extract	0.48	0.98	0.61	0.86	0.48	3.41	0.68
5.0% extract	0.36	0.61	0.72	0.33	0.35	2.36	0.47
Alpha-tocopherol	1.03	0.68	0.39	0.76	0.38	3.23	0.65
Pure Olive Oil	1.00	0.56	1.24	0.46	0.75	4.01	0.80

This result was analyzed using one-way ANOVA which revealed that there is no significant differences among the different treatments (P = 0.525)

Table 5. Diameter (in mm) of secondary blood vessels per 4 x 4 cm representative fractal segment of membrane chorioallantoic membrane of duck embryos treated with the test extract

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.46	0.43	0.45	0.84	0.28	2.46	0.49
0.06% extract	0.81	0.57	0.72	0.45	0.58	3.13	0.63
0.1% extract	0.78	0.47	0.72	0.49	0.64	3.10	0.62
1.0% extract	0.44	0.75	0.55	0.80	0.58	3.13	0.63
5.0% extract	0.37	0.88	0.54	0.40	0.38	2.57	0.51
Alpha-tocopherol	0.62	0.65	0.51	0.44	0.28	2.50	0.50
Pure Olive Oil	0.78	0.47	0.95	0.38	0.68	3.25	0.65

One-way ANOVA test of the data revealed that there is no significant difference among the different treatments (P = 0.615)

Table 6. Percent vascularity inhibition of the five concentrations of the ethanolic extract of Costus igneus and alpha-tocopherol (positive control)

Treatment	Total number of Blood Vessels Observed (PBV + SBV + TBV)	Percent Vascularity Inhibition
Positive Control (Alpha-Tocopherol)	76	18.28%
0.01 % extract	78	16.13%
0.06% extract	65	30.11%
0.1 % extract	73	21.51%
1.0% extract	77	17.20%
5.0 % extract	72	22.58%
Negative Control (Pure olive oil)	93	0.00%

Total of five replicates for each treatment

The percent vascularity inhibition was computed to assess the degree of inhibition of blood vessel formation exerted by crude ethanolic extract from Insulin plant (C. ignues) with reference to the negative control [20].

Table 7. Mortality rates of the embryos after administration of treatments

Treatments	Number of Embryos observed	Number of Dead Embryos				Total	Mortality Rate
		After 24 hrs.	After 48 hrs.	After 96 hrs.	After 240 hrs.		
0.01% extract	20	0	0	8	2	10	50%
0.06% extract	20	0	0	9	4	13	65%
0.1% extract	20	0	0	7	5	12	60%
1% extract	20	0	0	11	1	12	60%
5% extract	20	0	0	14	0	14	70%
Alpha-tocopherol	20	0	0	9	2	11	55%
Pure olive oil	20	0	0	7	4	11	55%

B. TERATOGENICITY ASSAY

Table 8. Data on body weights (in grams) of the duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	6.90	9.90	11.30	10.20	9.30	47.60	9.52
0.06% extract	8.60	6.70				15.30	7.65
0.1% extract	11.70	10.00	10.40			32.10	10.70
1.0% extract	10.10	8.10	7.60			25.80	8.60
Alpha-tocopherol	10.70	7.60	10.20	6.10		34.60	8.65
Pure olive oil	7.30	9.80	10.00	8.50		35.60	8.90

Blank means no data because the embryo died before the 18th day of incubation

The one-way ANOVA revealed that the effects exerted by the different treatments are not significantly different (P = 0.360)

Table 9. Data on body lengths (in mm) of the duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	86.74	84.19	74.27	83.12	79.46	407.78	81.56
0.06% extract	67.24	75.95				143.19	71.60
0.1% extract	81.62	90.24	84.66			256.52	85.51
1.0% extract	68.93	75.23	85.20			229.35	76.45
Alpha-tocopherol	83.56	87.40	80.60	57.09		308.65	77.16
Pure olive oil	76.98	80.33	78.31	75.90		311.52	77.88

Blank means no data because the embryo died before the 18th day of incubation

The one-way ANOVA revealed that the effects exerted by the different treatments are not significantly different (P = 0.424)

Table 10. Body mass index (in mg/mm2) of the experimental duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.92	1.4	2.05	1.48	1.47	7.32	1.46
0.06% extract	1.9	1.16				3.06	1.53
0.1% extract	1.76	1.23	1.45			4.44	1.48
1.0% extract	2.13	1.43	1.05			4.61	1.54
Alpha-tocopherol	1.53	0.99	1.57	1.87		5.96	1.49
Pure olive oil	1.23	1.52	1.63	1.48		5.86	1.46

Blank means no data because the embryo died before the 18th day of incubation

The one-way ANOVA revealed that the effects exerted by the different treatments are not significantly different (P = 1.000)

Table 11. Eye diameter (in mm) to body length (in mm) ratio of the duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.10	0.10	0.11	0.08	0.09	0.48	0.10
0.06% extract	0.10	0.12				0.22	0.12
0.1% extract	0.10	0.09	0.09			0.27	0.10
1.0% extract	0.10	0.11	0.09			0.21	0.11
Alpha-tocopherol	0.10	0.09	0.13	0.09		0.41	0.10
Pure olive oil	0.11	0.11	0.11	0.11		0.22	0.11

Blank means no data because the embryo died before the 18th day of incubation

The data when subjected to one-way ANOVA revealed that there are no significant differences among the different treatments (P = 0.485)

Table 12. Beak length (in mm) to body length (in mm) ratio of the duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.14	0.16	0.15	0.16	0.14	0.76	0.15
0.06% extract	0.12	0.17				0.29	0.15
0.1% extract	0.18	0.16	0.13			0.47	0.16
1.0% extract	0.16	0.13	0.13			0.42	0.14
Alpha-tocopherol	0.14	0.14	0.15	0.19		0.62	0.15
Pure olive oil	0.16	0.15	0.17	0.20		0.68	0.17

Blank means no data because the embryo died before the 18th day of incubation

The result of the one-way ANOVA test revealed that there are no significant differences among the different treatments (P = 0.535)

Table 13. Neck length (in mm) to body length (in mm) ratio of the duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.28	0.26	0.27	0.28	0.19	1.27	0.25
0.06% extract	0.28	0.26				0.54	0.27
0.1% extract	0.23	0.25	0.22			0.69	0.23
1.0% extract	0.34	0.19	0.27			0.53	0.27
Alpha-tocopherol	0.27	0.19	0.23	0.20		0.88	0.22
Pure olive oil	0.27	0.22	0.22	0.21		0.49	0.25

Blank means no data because the embryo died before the 18th day of incubation

The data were analyzed using the one-way ANOVA test which revealed that there are no significant differences among the different treatments (P = 0.546)

Table 14. Forelimb length (in mm) to body length (in mm) ratio of the duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.39	0.41	0.39	0.32	0.35	1.85	0.37
0.06% extract	0.44	0.39				0.83	0.42
0.1% extract	0.39	0.41	0.39			1.18	0.39
1.0% extract	0.46	0.41	0.36			0.86	0.41
Alpha-tocopherol	0.37	0.32	0.40	0.37		0.69	0.36
Pure olive oil	0.37	0.35	0.40	0.40		0.73	0.38

Blank means no data because the embryo died before the 18th day of incubation

The one-way ANOVA test revealed that there are no significant differences among the different treatments (P = 0.366)

Table 15. Hind limb length (in mm) to body length (in mm) ratio of the duck embryos after 18 days of incubation in different treatments

Treatments	Replicates					Total	MEAN
	1	2	3	4	5		
0.01% extract	0.45	0.50	0.44	0.38	0.47	2.25	0.45
0.06% extract	0.47	0.44				0.90	0.45
0.1% extract	0.45	0.44	0.45			1.34	0.45
1.0% extract	0.43	0.46	0.46			1.35	0.45
Alpha-tocopherol	0.40	0.33	0.39	0.48		1.60	0.40
Pure olive oil	0.42	0.45	0.49	0.44		1.81	0.45

Blank means no data because the embryo died before the 18th day of incubation

One-way ANOVA test revealed that there are no significant differences among the different treatments (P = 0.428)

VII. RESULTS AND DISCUSSION

Chorioallantoic Membrane Vascularity Assay (CAM)

The potential anti-angiogenic activity of Insulin plant (*C. igneus*) leaves was assessed using the CAM model of *A. platyrhynchos* embryos. Quantitative analysis revealed that there is no statistical significance on any of the concentrations from the test plant.

According to [19], the major physiological activity of the vitamin E is its anti-oxidant capacity. Alpha-tocopherol has the highest biological activity among vitamin E isomers. [15] have demonstrated that alpha tocopherol can inhibit angiogenesis through inhibiting the release of vascular endothelial growth factor (VEGF) from human tumor cells. In the study of [21], they have demonstrated that alpha-, gamma- and delta tocopherols can reduce inflammatory angiogenesis in human microvascular endothelial cells.

Phytochemicals from *C. igneus* leaves showed that it is rich in protein, iron, and antioxidant components such as ascorbic acid, α-tocopherol, β-carotene, terpinoids, steroids, and flavonoids. It was showed in another study that methanolic extract was found to contain the highest number of phytochemicals such as carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, and flavonoids [5].

This suggest that there are common angiosupressive phytochemicals between Myra-E (alpha-tocopherol) and Insulin plant (*C. igneus*) such as flavonoids, anti-oxidants and alpha-tocopherol itself are components of Insulin plant. Thus, there is an assumption that the result will be positive for anti-angiogenic effect but as the result suggest that the different treatment has an insignificant effect using Chorioallantoic Membrane Vascularity Assay (CAM).

Teratogenicity Assay

The three basic standards of developmental toxicity by teratogens are delayed growth of organ systems, growth retardation and the most severe, birth defects in live offspring. In this study, the crude ethanolic extract of Insulin plant (*Costus igneus*) was also evaluated for possible teratogenic effects on body weight, body length, body mass index (BMI), beak length, eye diameter, neck length, forelimb length and hind limb length of *Anas platyrhynchos* embryos [10].

The test extracts did not significantly affect body weight, body length, body mass index, beak length, eye diameter, neck length, forelimb length and hind limb length of *Anas platyrhynchos* embryos. Thus, the plant extract of *C. igneus* has no teratogenic effect.

VIII. CONCLUSION AND RECOMMENDATIONS

Conclusion

Based on the analysis of the data using Kruskal-Wallis test and one-way analysis of variance (ANOVA) it can be concluded that the crude ethanolic extract of Insulin plant (*Costus igneus*) has no angiosuppresive influence through the chorioallantoic membrane (CAM) vascularity in mallard duck (*Anas platyrhynchos*) embryos. The plant extract did not affect the body weight, body length, body mass index, eye diameter, beak length, neck lengths, forelimbs and hind limbs. Therefore, Insulin plant (*Costus igneus*) has no teratogenic effect.

Recommendations

1. Similar study using stronger positive control to evaluate differences.A phytochemical analysis of the plant extract to know its bioactive components.
2. Identification and characterization of the anti-angiogenic and teratogenic components of the ethanolic extract of Insulin (*Costus igneus*).
3. Similar study but more high-tech and sophisticated equipment for incubation to attain proper heat distribution.
4. Similar study with much more replicate to have more accurate statistical results
5. Studies using different *in vitro* types of assay (e.g. aortic ring assay, cell proliferation assay) to test other anticancer property of the crude ethanolic extract aside from angiogenesis inhibition.
6. Similar study using other members of family Costaceae.

IX. PLATES

Fig. 1Appearance of Image J software version 1.45s

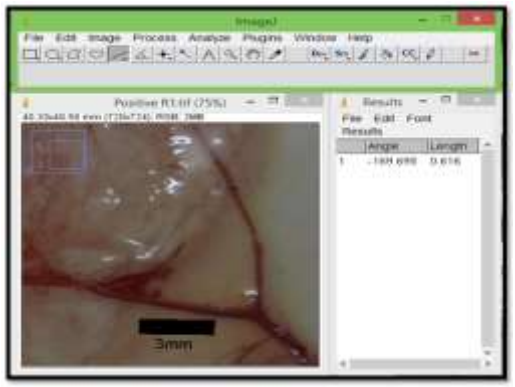


Fig. 2 Representative photograph of CAM vascular area of embryos (A) treated with alpha-tocopherol (positive control) showing the reference point (black arrow) which is the primary blood vessel nearest to the heart, and the 4 x 4 cm representative fractal segment (B). Arrows indicate primary (orange); secondary (violet); and tertiary (light green) blood vessels

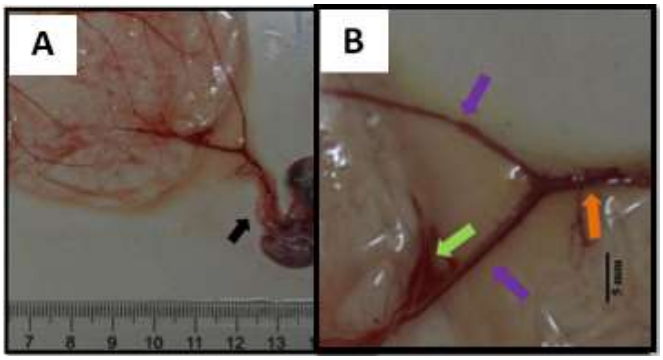


Fig. 3 Representative photographs of 4 x 4 cm fractal segments of CAM vascular area in treated embryos (A) Treated with pure olive oil; (B) Treated with alpha-tocopherol; (C) Treated with 0.01% extract; (D) Treated with 0.06% extract; (E) Treated with 0.1% extract; (F) Treated with 1.0% extract (G) Treated with 5.0% extract.

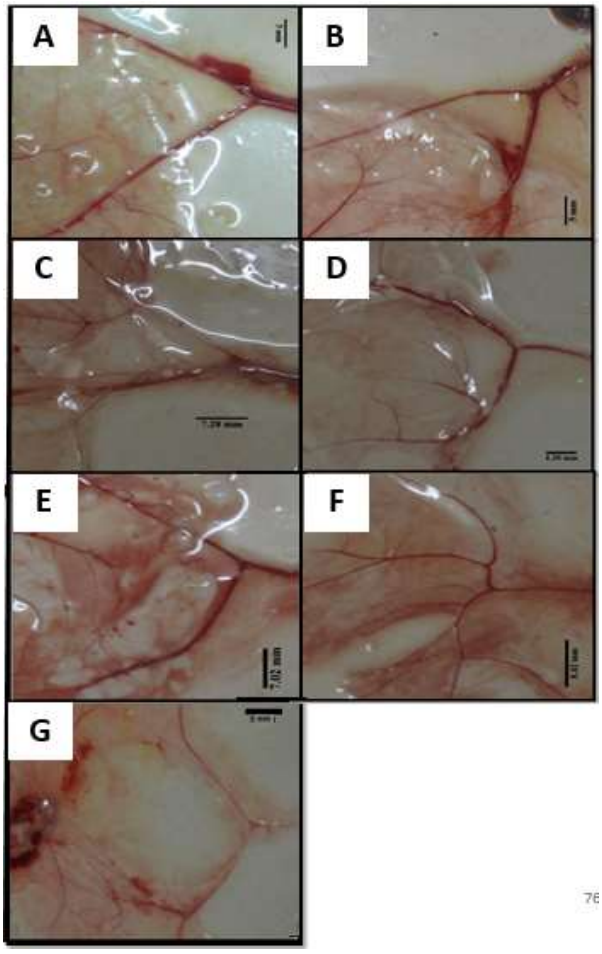


Fig. 4 Representative photographs of embryos (day 18)

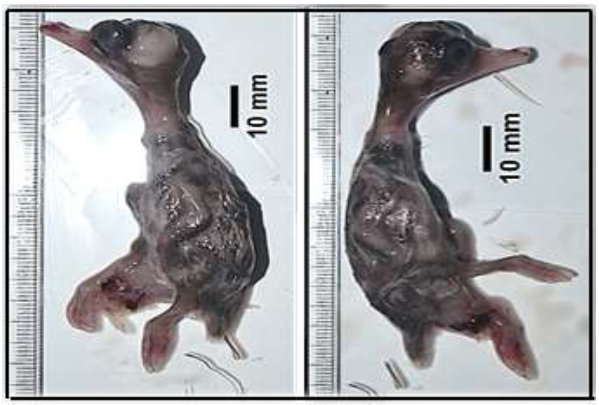
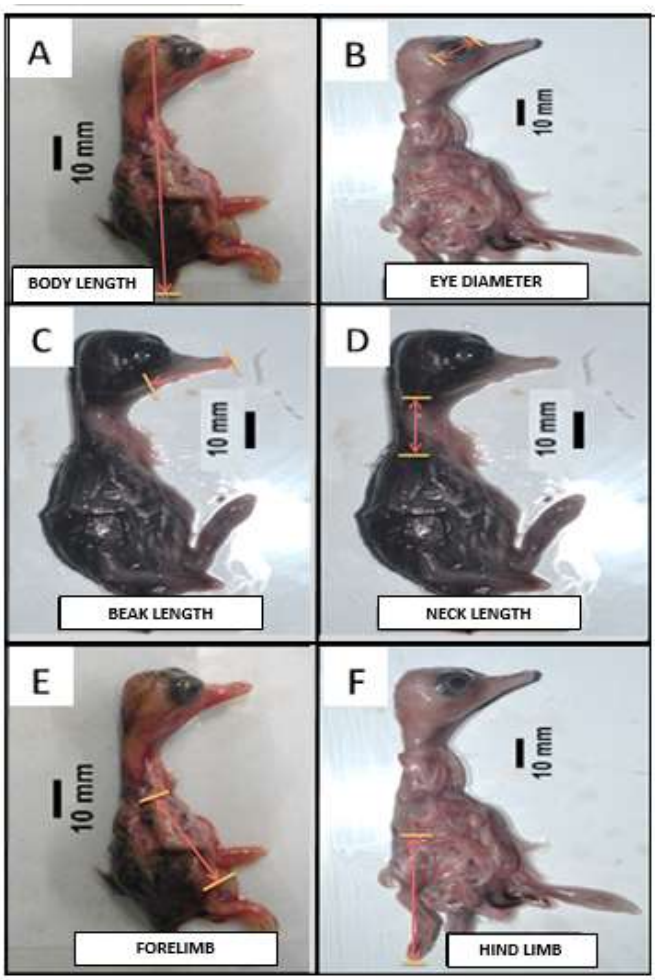


Fig. 5 Morphometric features of A. platyrhynchos embryos in 18 days of incubation: (A) body length, (B) eye diameter, (C) beak length, (D) neck length, (E) forelimb and (F) hind limb.



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