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Selected Philippine Herbal plant extracts as Angiogenesis inhibitors using Chick Chorioallantoic Membrane (CAM) assay

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Abstract

The study was conducted to assess the phytochemical components and properties of herbal plant extracts, such as (a) sabungai (Gynura nepalensis DC), (b) pandan (Pandanus odoratissimus L.) and (c) tsaang-gubat (Carmona retusa (Vahl.) Masam.), as potential angiogenesis inhibitors using the Chick Chorioallantoic Membrane (CAM) assay. Phytochemical analysis showed that the three extracts were found to have alkaloids, tannins and flavonoids. Only pandan extract was assessed to contain anthraquinones and steroids. The treatments were individually compared to the positive control and negative control of the study. Statistical analysis showed significant difference on the mean number of branching points of eggs treated sabungai extract, pandan extract and tsaang-gubat as compared to negative control of the study. Sabungai extract was found significantly different to the positive control. On the other hand, no significant difference is seen in the mean number of the branching points of the eggs treated with pandan and tsaang-gubat extracts when individually compared to the positive control. Using the formula for CAM Vascularity that determines the inhibition properties of the extracts, it was found out that tsaang-gubat gained the highest inhibition of vascularity followed by pandan, then sabungai.

Keywords: Anti-angiogenesis, *Gynura nepalensis DC, Pandanus odoratissimus, Carmona retusa (Vahl.) Masam.,* vascularity, CAM, pandan, gynura, tsaang-gubat, angiogenesis.

Introduction

Angiogenesis is a normal and vital process in growth and development, as well as in wound healing and in the formation of granulation tissue[1,2]. This is a physiological process of new blood vessel development from pre-existing capillaries. However, this complex multi-step process is a main prerequisite for tumor growth and plays an important role in tumor invasion and metastasis[1]. Thus, it may lead to cancer, which is known as a fatal genetic disease. However, angiogenesis inhibitors can be used to impede the abnormal growth of blood vessels.

Nowadays, many researchers are studying the tea *Camellia sinensis,* because of its promising properties in the field of medicine and its vast availability in the market sold as instant tea, milk tea, yoghurt tea and other products. One of the major components of this tea is polyphenol which is considered as potential angiogenesis inhibitor. Tea preparations and tea polyphenols have been shown to inhibit formation and growth of a variety of solid tumors in laboratory rats and mice, thus, can reduce the risk of cancer in rectum, colon and pancreas[3]. Polyphenol is rich in catechin, especially Epigallocatechin gallate (ECGC) and is a potent antioxidant.

In the Philippines, however, there were few studies being conducted among our herbal plants with regards to their potentials as angiogenesis inhibitors. Of the many native herbal plants locally known are tsaang- gubat (*Carmona retusa (Vahl.) Masam.*), sabungai *(Gynura nepalensis DC)* and pandan *(Pandanus odoratissimus L.).* Researches have been focused only on the phytochemistry of these plants. Hence, this study aimed to evaluate the angiogenesis inhibitor potentials of these selected herbal plants using the Chick Chorioallantoic Membrane (CAM) Assay.

Material and Methods

Research Design: The study utilized the experimental method of research to assess the selected herbal plants as potential angiogenesis inhibitors using the Chick Chorioallantoic Membrane (CAM) Assay. Ten fertilized chicken eggs per treatment were analyzed. The herbal plants used in the study were: i. Sabungai *(Gynura nepalensis DC); ii.* Pandan *(Pandanus odoratissimus L.);* and *iii.* Tsaang-gubat (*Carmona retusa (Vahl.) Masam.*).

Research Procedure: Preparation of Cultivar: For each cultivar, dried leaves (1000g) were grounded and placed in dark-colored amber bottles with sufficient amount of ethanol (just enough to submerge the materials). The plant materials were soaked for 48 hours and filtered with a clean and fine filter paper. The filtered samples were then concentrated with a rotary evaporator. The extracts were stored in tightly stoppered containers in the refrigerator under 5^{o}C. Each container was labelled with the name of the plant.

Phytochemical Screening: Phytochemical components were assessed using the test tube method. The compounds that were

tested are flavonoids, tannins, saponins, alkaloids, steroids, and anthraquinones.

Chick Chorioallantoic Membrane (CAM) Assay[4-6]: Incubation of Eggs. Chicken fertilized eggs were incubated at 37^{O}C in a standard cell culture incubator with 65% relative humidity to avoid dehydration of the eggs, cracking and damage to the egg shell up to Day 10.

Drilling of Hole on the Eggs: On the 10^{th} day, the surface of the eggs were disinfected and 1x1 holes were drilled at the blunt ends of the shell.

Abscission of the Eggs: Extracts of the cultivars (0.03 ml) were drawn and transferred on the wells of a titer plate. The sterilized filter paper discs were placed on each cell and were left covered. Ten days after soaking the filter paper disc with the extracts, egg membranes were abscised and treatments were applied directly to the CAM via absorption onto the filter paper disc. A paper clinical tape was used to cover the manipulation window. Eggs were returned to the incubator for another two days.

Visual Assessment of the CAM: On the 12^{th} day of incubation, reactions of the CAM were observed. CAM was harvested carefully by the removing hard shell leaving intact the soft membrane. The shell less embryo was transferred to a petri dish. The number of branching points of blood vessels was manually counted. The blood vessels were photographed using a Canon 600D DSLR.

Statistical Treatment: Data were subjected to Analysis of Variance (ANOVA) of the Complete Randomized Design (CRD) and Duncan's Multiple Range Test (DMRT). The branching point of each cultivar was individually compared to positive and negative control of the study.

Results and Discussion

Phytochemical Components of Selected Herbal Plants: Phytochemical components of sabungai, pandan and tsaang-gubat are shown in table-1. Sabungai extract was seen to contain alkaloids, catechic tannins, saponins and flavonoids. No steroids and anthraquinones were observed in the test. An ethanolic extract of the plant leaves yielded alkaloids and volatile oils[7]. On the other hand, pandan extract contained phenolics compounds and alkaloids[8]. In addition, tsaang-gubat's phytochemical components are alkaloids, tannins and flavonoids[9,10].

Among the phytochemical components, phenolic compounds such as flavonoids and tannins are responsible for the angiogenic inhibition activity of plants. Flavonoid was found to have various effects on biochemical pathways such as anti-inflammation, inhibition of cell proliferation, antioxidation, detoxification of mutagenic metabolites, apoptosis and inhibition of angiogenesis and can serve as chemopreventive agent in numerous epidemiological studies[11]. It has also been shown to inhibit angiogenesis and the proliferation of tumor cells and endothelial cells in vitro[12]. Moreover, tannic acid or tannin inhibits angiogenesis induced by human cholangiocarcinoma cells as studied in a study. Tannic acid inhibited VEGF mRNA and protein expression in Mz-ChA-1 human cholangiocarcinoma cells, reduced tumor cell induced endothelial cell proliferation in vitro and decreased angiogenesis as well as xenograft neovascularity and growth in vivo. The anti-tumoral effects of tannic acid may involve inhibition of tumor-cell induced angiogenesis through blocking the induction of VEGF[13].

Branching Points of Eggs treated with Sabungai, Pandan, and Tsaang-Gubat extract: The mean number of the branching points of the sabungai, pandan and tsaang-gubat are seen in table-2, figure-1. Based on statistical analysis, there is a significant difference (f-value=254.552) in the mean number of branching points of eggs treated with sabungai extract (43.00) as compared to those of the negative control (189.93) and the positive control (20.23).

The mean number of branching points of eggs treated with pandan extract (28.53) as compared to that of the negative control (189.93) also showed a significant difference (f-value=406.99). On the other hand, the average mean number of the branching points of eggs treated with pandan extract was comparable to the mean number of branching points of the positive control (20.23).

Table-1
Phytochemical Components of the Selected Herbal Plants

	Tsaang-gubat *(Carmona retusa (Vahl.) Masam.)*	**Pandan** *(Pandan odoratissimus L.)*	**Sabungai** *(Gynura nepalensis DC)*
Alkaloids	Present	Present	Present
Tannins	Present	Present	Present
Flavonoids	Present	Present	Present
Steroids	None	Present	None
Anthraquinones	None	Visible red coloration	None

Based on the Analysis of Variance and Duncan's Multiple Range Test, the mean number of branching points of eggs

treated with tsaang-gubat (20.17) revealed a significant difference (f-value=536.83) when compared to the mean number of branching points of eggs in the negative control (189.93); yet, it also showed comparable angiogenesis inhibition activity with the positive control (20.23). The phenolic compounds seen on the phytochemical test conducted in the study may support the inhibition of angiogenesis in the CAM. Phytochemicals including flavonoids, sulphated carbohydrates, triterpenoids, catechols, tannins and aromatic substances have been reported to have anti-angiogenic effects[11].

Tea cultivars can suppress VEGF that may lead to fewer blood vessels[12]. It was also revealed that flavonoids have been shown to inhibit angiogenesis and proliferation of tumor cells and endothelial cells in vitro and can inhibit vascular endothelial growth factor (VEGF)-induced cell proliferation and migration in HUVECs[14].

Of the three cultivars, Sabungai extract had the highest mean number of branching points (43.00) followed by pandan extract (28.53) , and tsaang-gubat extract with the least mean number of branching points (20.17). Based on statistical analysis, the mean number of the branching points of eggs treated with sabungai, pandan and tsaang-gubat are significantly different with each other. Tsaang-gubat and pandan extracts have comparable anti-angiogenic effects. While, pandan and sabungai extracts showed comparable effects in terms of the inhibition of blood vessels. They were able to lower down the number of blood vessels when compared to the negative control. This indicates that the herbal extracts have anti-angiogenesis potential.

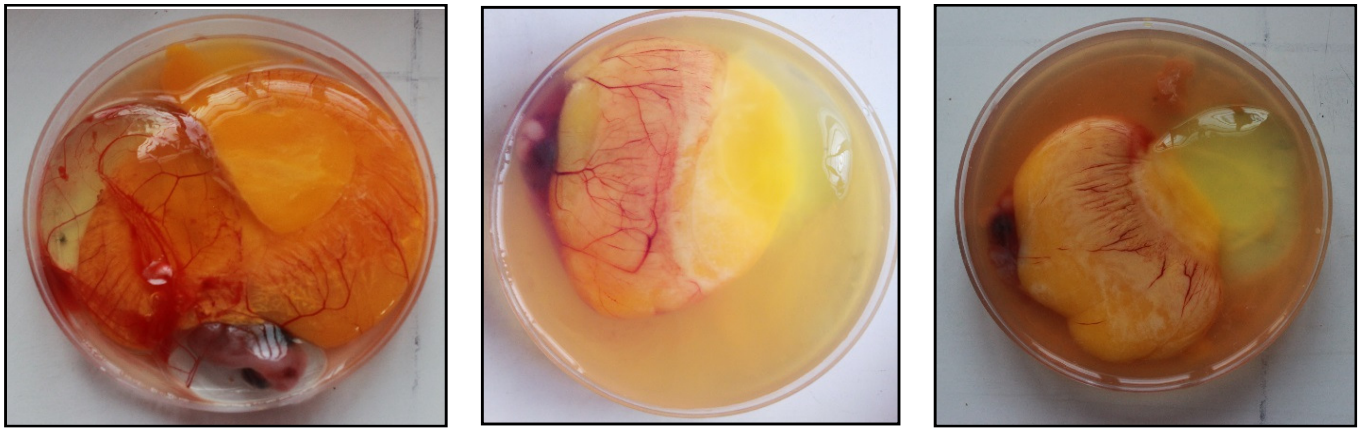
Cultivars contain polyphenolic compounds, which include flavanols, flavandiols, flavonoids, and phenolic acids[15-18]. These phenolic phytochemicals helped on the biochemical pathways such as anti-inflammation, inhibition of cell proliferation, antioxidation, detoxification of mutagenic metabolites, apoptosis and inhibition of angiogenesis[11].

CAM Vascularity of the Selected Herbal Plants: The average mean number of branching points of the cultivars were assessed for CAM Vascularity. Based on the computed data using the formula for CAM Vascularity, the percentages as shown on table-3, figure-2 revealed that the vascularity of the herbal plant extracts decreased when compared with the negative control. Tsaang-gubat had the highest inhibition of vascularity with -89.05%. It was followed by pandan with -85.26% while sabungai, with the least number of branching points with -77.09%.

Table-2
Branching Points of the Herbal Plants

	Mean Number	F-value	SD
Retinoic Acid (Positive Control)	20.23	-	-
Distilled Water (Negative Control)	189.93	-	-
Sabungai Extract *(Gynura nepalensis DC)*	43.00[b]	6.828	29.85829
Pandan Extract *(Pandan odoratissimus L.)*	28.53[ab]	-	25.31148
Tsaang-gubat (*Carmona retusa (Vahl.) Masam.)*	20.17[a]	-	15.05412

Legend: ** - Significant at 1% level



a b c

Figure-1
Branching Points of the Eggs treated with Herbal Plants. (a) T1 - Egg treated with Sabungai(b) T2 - Egg treated with Pandan(c) T3 - Egg treated with Tsaang-gubat

Decreased vascularity of the primary tumor is virtually always associated with decreased formation of metastatic colonies[2]. Chances are, the decrease in the vascularity also decrease metastasis.

The inhibition of angiogenesis is done by suppressing the action of the vascular endothelial growth factor of VEGF since it is considered as one of the most important proangiogenic factors. VEGF also potentiates microvascular hyperpermeability, which can both precede and accompany angiogenesis[19]. Phenolic compounds such as flavonoids and tannic acid may have helped in blocking the induction of VEGF[11,13].

Table-3
CAM Vascularity of the Herbal Plants

	CAM Vascularity
Sabungai Extract *(Gynura nepalensis DC)*	-77.09%
Pandan Extract *(Pandan odoratissimus L.)*	-85.26%
Tsaang-gubat (*Carmona retusa (Vahl.) Masam.)*	-89.05%

Conclusion

Based from phytochemical screening of the herbal plants, it was observed that sabungai, pandan and tsaang-gubat extracts have alkaloids, tannins, and flavonoids and only pandan extract contains anthraquinones and steroids. Statistical analysis showed significant difference on the mean number of branching points produced by the three herbal plant extracts when compared to negative control. Sabungai extract was found to be significantly different to the positive control. On the other hand, no significant difference was seen in the mean number of the branching points of the eggs treated individually with pandan and tsaang-gubat extracts when compared to the positive control. The mean number of branching points of the eggs treated with herbal plant extracts were significantly different with each other. Based on the formula for CAM vascularity, tsaang-gubat had the highest inhibition of vascularity, followed by pandan and sabungai got the least number of branching points. Hence, the three cultivars showed promising potential as angiogenesis inhibitors, yet further clinical studies should be observed.

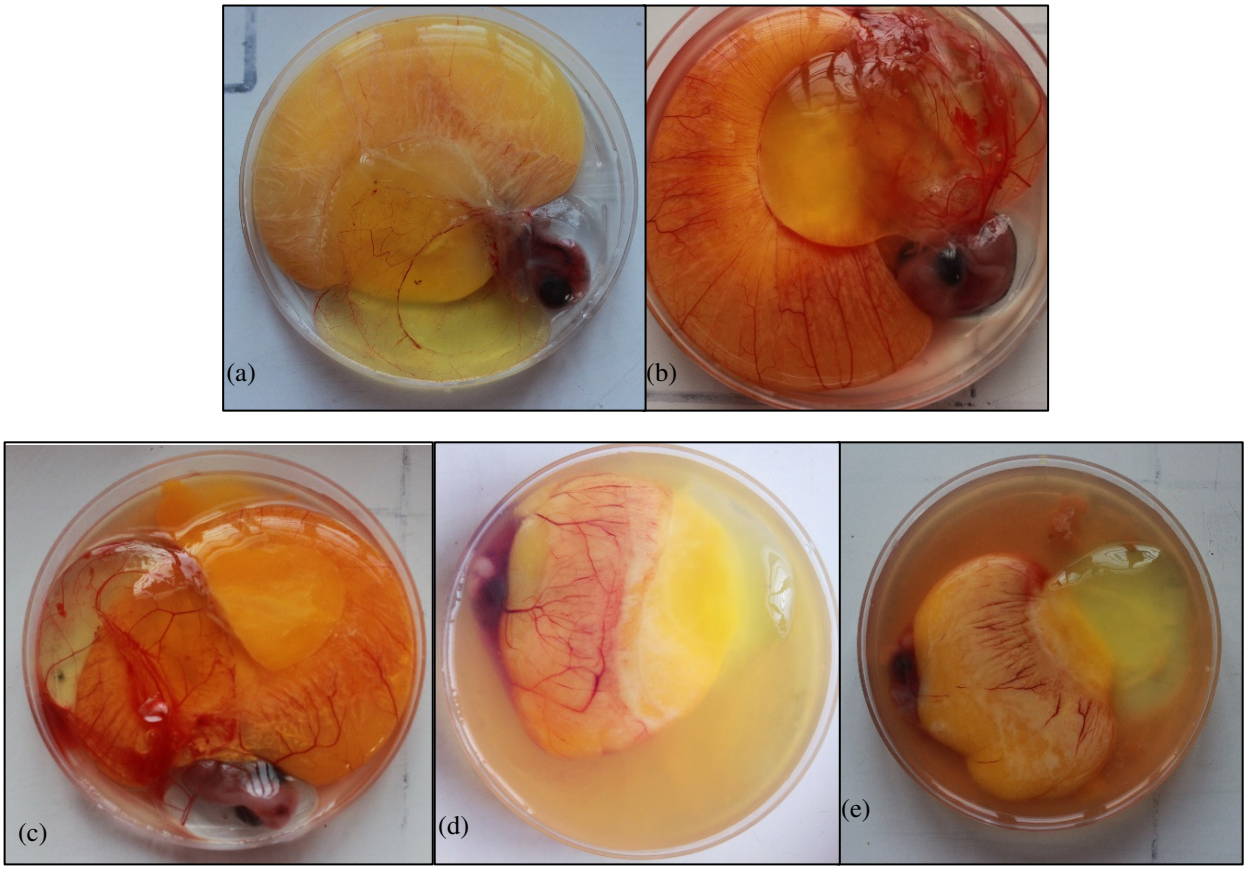


Figure-2
CAM Vascularity of the Treatments of the Study. (a) T+ - Egg treated with RA, (b) T- - Egg treated with distilled water, (c) T1 - Egg treated with sabungai, (d) T2 - Egg treated with pandan, and; (e) T3 - Egg treated with tsaanggubat

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