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Research Paper

Anti-Angiogenic Activity of Sabungai (*Gynura Procumbens*) using a Cancer-induced Chicken Embryo

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Abstract

Cancer has been increasing over recent years and is considered the primary cause of mortality following cardiovascular disease. Angiogenesis, the biological process by which new blood vessels are generated, plays a crucial role in the growth and spread of tumors. The objective of this study was to assess the anti-angiogenic activity of different Sabungai (*Gynura procumbens*) leaf extract concentrations in the *Chorioallantoic Membrane* (CAM) of cancer-induced chicken embryos. The results of a one-way analysis of variance (25.20 ± 2.70, 27.20 ± 1.08, and 22.80 ± 1.66) indicated a statistically significant difference in CAM vascularization between the various treatments. Post hoc analysis utilizing Tukey's Honest Significant Difference (HSD) test revealed that Treatment 2 (300ppt) significantly affected CAM vascularization, as evidenced by an average vascularization count of 27.20 ± 1.08, followed by Treatment 1 (100ppt) and Treatment 3 (500ppt). The results indicated that the extract from Sabungai leaves effectively hinders the formation of new blood vessels in a dose-dependent manner. The highest concentration of leaf extract (500 ppt) resulted in an average vascularization of 22.80 ± 1.66. However, excessively high doses can cause bleeding and embryo death. The results demonstrated that sabungai leaf extract affected blood vessel development in chicken embryos. The results of this research have various implications that contribute to scientific knowledge and potential applications in cancer treatment.

Keywords: *angiogenesis, cancer, chorioallantoic membrane, Gynura procumbens*

INTRODUCTION

Cancer is a leading global health crisis, second only to heart disease in causing deaths (Nurhidayati et al., 2021). The incidence of cancer is increasing annually (Taiwo et al., 2017), as highlighted in the Global Cancer Statistics 2024. Understanding cancer's growth mechanisms, especially angiogenesis, is crucial for developing effective treatments. Angiogenesis, the formation of new blood vessels from pre-existing blood vessels, plays a significant role in tumor growth and metastasis by supplying oxygen and nutrients to cancer cells (Gupta et al., 2022).

Angiogenesis supports tumor growth and spread by enabling cancer cells to invade adjacent tissues and form new colonies (Nurhidayati et al., 2021). Suppressing angiogenesis can starve tumors of the necessary nutrients, potentially arresting cancer progression (Lopes-Coelho et al., 2021). The *Chorioallantoic Membrane* (CAM) assay in chicken embryos is an effective and cost-efficient model for studying angiogenesis and testing the antiangiogenic properties of substances (Kennedy et al., 2021). The unique properties of *G. Procumbens* make it a promising candidate for anti-angiogenic research.

Previous studies have shown that plant extracts possess significant antiangiogenic effects, suggesting that natural compounds could be valuable alternatives to synthetic therapies. Traditional medicinal plants like *Gynura Procumbens* are used in Southeast Asia to treat various

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ailments, including cancer (Afandi et al., 2014). *G. Procumbens* is known for its anti-inflammatory, anti-hyperlipidemic, and anti-hypoglycemic properties (Zhang & Tan, 2000; Bohari et al., 2000). Despite its extensive use, its antiangiogenic potential remains unexplored.

This study investigated the antiangiogenic effects of different concentrations of G. procumbens leaf extract in an experimental setup using cancer-induced chicken embryos. The findings could provide a basis for developing new cancer treatments and offer an educational tool for integrating real-world scientific research into STEM curricula. Engaging students in such research can enhance their understanding of biological processes and the potential applications of herbal medicines and foster critical thinking and scientific creativity (Kırıcı & Bakırcı, 2021).

Objectives of the Study

This study aimed to determine the anti-angiogenic activity of *G. Procumbens* leaf extract in cancer-induced chicken embryos, specifically determining the total number of vascularization of the *Chorioallantoic Membrane* (CAM) in three different *G. Procumbens* leaf extract treatments.

RESEARCH METHOD

The antiangiogenic potential of *G. Procumbens* was evaluated using an experimental design. The collection and experiments were conducted from March to April 2024.

Collection and Extraction of Plant Material

Fresh leaves of *G. Procumbens* were collected and harvested from Daine Indang, Cavite. The petiole and midrib were removed, and the leaves were washed with distilled water to remove dirt and impurities. The leaves were air-dried until they became brittle and then powdered using a blender. The 500 g powdered leaves of *G. Procumbens* were soaked in 95% ethanol for 48 h in a cool dark place. The leaf mixture was then filtered, and the filtrate was evaporated to obtain pure crude extract and stored in a refrigerator at 5-10°C.

Different concentrations of *G. Procumbens* extract were prepared using the dilution method, in which 1 ml/L extract was diluted in separate test tubes, including the additional distilled water, with treatments ranging from Treatment 1 (100 ppt) to Treatment 2 (300 ppt) and Treatment 3 (500 ppt). The dilution factor was obtained by dividing the final volume by the initial volume.

Angiogenic Assay

Seventy-five 10-day-old fertilized chick eggs were obtained from Sustamina Chavez Farm, Silang, Cavite, and incubated for 48 h at 37°C–39°C and 50%–60% humidity. The *G. Procumbens* extract was administered to the eggs' *Chorioallantoic Membrane (CAM)* using a sterile syringe at specific volumes for each treatment group. Tests were performed on the 10th day of egg incubation. The experimental design consisted of a control and treatment groups. The control groups consisted of negative controls administered with distilled water and positive controls administered with pure *G. Procumbens* leaf extract. The treatment group was administered *G. Procumbens* leaf extract at concentrations of 100, 300, and 500 ppt. All treatments were tested on the CAM of chicken embryos maintained in an incubator at 37°C–39°C with 50%–60% humidity for 48 h.

Observation and Data Collection

Observations were conducted after 48 h of incubation. The embryos were transferred to a Petri dish, and the soft membrane was extracted and observed from the embryo. The observation was conducted via photo observation, where the treated CAMs were captured using a camera, and the number of collaterals at the points of intersection of the blood vessels of the CAM of eggs treated with *G. Procumbens* and with benzene solution (10%) was counted and compared (Oktavia et al., 2017).

Statistical Analysis

The number of new blood vessel points was counted, and the mean and standard deviation were determined. Statistically significant differences were determined using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test, where $P<0.05$ was considered statistically significant.

FINDINGS AND DISCUSSION

This study investigated the antiangiogenic activity of Sabungai leaf extract on the vascularization of the chorioallantoic membrane of chicken embryos (CAM).

Table 1. Total number of vascularizations of the *Chorioallantoic Membrane* (CAM) in three different treatments of *G. Procumbens* leaf extract

Treatments	Amount/Concentration	Mean	SD
Treatment 1	0.3 ml of 100ppt GP extract	28.00	2.121
	0.4 ml of 100ppt GP extract	24.40	0.894
	0.5 ml of 100ppt GP extract	23.20	2.168
Treatment 2	0.3 ml of 300ppt GP extract	27.80	0.837
	0.4 ml of 300ppt GP extract	27.20	1.304
	0.5 ml of 300ppt GP extract	26.60	0.894
Treatment 3	0.3 ml of 500ppt GP extract	24.00	0.707
	0.4 ml of 500ppt GP extract	23.00	1.414
	0.5 ml of 500ppt GP extract	21.40	1.673
Negative Control	0.3 ml DO water	14.00	1.225
	0.4 ml DO water	12.40	2.510
	0.5 ml DO water	11.80	1.483
Positive Control	0.3 ml of GP pure extract	18.20	1.304
	0.4 ml of GP pure extract	19.00	1.414
	0.5 ml of GP pure extract	18.80	1.304

Legend: GP-Gynura procumbens DO-Distilled Water

Vascularization was reduced at higher extract concentrations (Table 1). Treatment 1 (100 ppt) had an average vascularization of 24.40, 23.20, and 28.00 mmHg; Treatment 2 (300 ppt) produced an average vascularization of 27.80, 27.20, and 26.60 mmHg; and Treatment 3 (500 ppt) produced 24.00, 23.00, and 21.40 mmHg, respectively. The formation of new blood vessels in the negative control (distilled water) was the lowest at 14.00, 12.40, and 11.80. In contrast, the positive control, which consisted of pure *G. Procumbens* extract, exhibited intermediate levels at 18.20, 19.00, and 18.80. These results indicated a dose-dependent decrease in vascularization with increasing concentrations of *G. Procumbens* leaf extract (Nurhidayati et al., 2021; Oktavia et al., 2017), contrasting with the reduced angiogenesis observed in the negative control and the moderate response observed in the positive control.

Table 2. Differences in the number of CAM vascularizations among the three *G. Procumbens* leaf extract treatments

Treatments	No. of New Blood Vessels (Mean ± SD)	F (2,42)
Treatment 1	25.20 ± 2.70	
Treatment 2	27.20 ± 1.08	19.45 **
Treatment 3	22.80 ± 1.66	
(-) Control	14.00 ± 1.94	
(+) Control	18.67 ± 1.29	

Legend: Treatment 1 (100ppt), Treatment 2 (300ppt), and Treatment 3 (500ppt)
** - Test is significant @ p-value<0.01; * - Test is significant @ p-value<0.05

The results of the treatments indicated that benzene-induced chick embryos (25.20 ± 2.70, 27.20 ± 1.08, and 22.80 ± 1.66) increased the formation of new blood vessels (Table 2), suggesting that benzene applied to CAM imitates the role of cancer tissue as a control agent in angiogenesis compared with vascularization in the negative control group (14.00 ± 1.94).

Table 2 shows the number of CAM vascularizations (25.20 ± 2.70, 27.20 ± 1.08, and 22.80 ± 1.66) among the three treatments (100 ppt, 300 ppt, and 500 ppt) of *G. Procumbens* leaf extract. The result was consistent with that of a previous study, which exhibited anti-angiogenic activity, as the treatment prevented the formation of new blood vessels on fertilized chicken eggs and inhibited the expression of vascular endothelial growth factor (VEGF) (Tan et al., 2016). The combination of flavonoids, saponins, and tannins in the extracts may have inhibited the growth factor. A previous study found that methanolic extracts of jackfruit seeds induce apoptosis in cell culture. This may be due to the inhibition of endothelial cell migration and the proliferation of new blood vessels. Flavonoid compounds in jackfruit seeds overpower growth factors without damaging established endothelial cells (Oktavia et al., 2017).

Various concentrations of *G. Procumbens* leaf extract (100 ppt, 300 ppt, and 500 ppt) are effective angiogenesis inhibitors and have significant implications for cancer treatment. The study revealed that the F- value (19.45) was higher than the critical value (5.18) at the 0.01 significance level, indicating a significant difference in the CAM angiogenesis of the different experimental treatments. This finding suggested that treatment with different *G. Procumbens* leaf extracts significantly affected CAM vascularization in cancer-induced chicken embryos. In this study, *G. Procumbens* leaf extract also displayed anti-angiogenesis responses in the CAM of chicken embryos. The findings indicated that higher concentrations of *G. Procumbens* leaf extract inhibit the growth of new blood vessels in the CAM of chicken embryos, consistent with previous studies that observed decreased blood vessel growth at higher concentrations (Nurhidayati et al., 2021). In this study, the highest concentration of leaf extract (500 ppt) resulted in an average vascularization of 22.80 ± 1.66. However, excessively high doses can cause bleeding and embryo death (Nurhidayati et al., 2021). Similarly, previous studies using methanolic extracts from jackfruit seeds Oktavia et al. (2017) have shown that higher concentrations generally lead to more significant angiogenesis inhibition. However, concentrations above 52.50 μg/mL were toxic to the embryos. Inhibition of the formation of new blood vessels results in depriving tumor cells of nutrients and death (Nurhidayati et al., 2021).

The angiogenic activity of all treatments was significantly different. Hence, post hoc analyses were conducted using Tukey's Honest Significant Difference (HSD) test to comprehensively investigate these differences. The post hoc analysis demonstrated that Treatment 2 significantly affected CAM vascularization, as evidenced by an average vascularization count of 27.20 (SD =

1.08), followed by Treatments 1 and 3. Treatment 2 exhibited the greatest efficacy in reducing vascularization, suggesting its potential as a powerful antiangiogenic agent. Treatment 2 regularly exhibited a substantial decrease compared with the other treatments (Table 2), highlighting its potential as the most effective antiangiogenic concentration. The significant decrease in CAM counts observed in all treatments compared with the control suggests that *G. Procumbens* leaf extract exhibits anti-angiogenic activity, effectively hindering the formation of blood vessels.

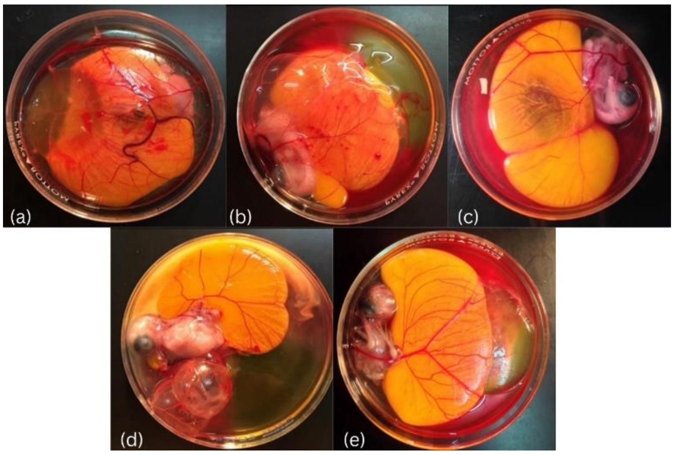

Figure 1. Images of CAMs of chicken embryos. (a) Distilled water as a negative control; (b) 100 ppt GP leaf extract + 10% benzoene; (c) 300 ppt GP leaf extract + 10% benzoene; (d) 500 ppt GP leaf extract + 10% benzoene; (e) pure GP leaf extract + 10% benzoene as a positive control

Phytol, which is found in *G. Procumbens*, can kill specific cancer cell lines, including those from melanoma, cervical cancer, prostate adenocarcinoma, and colorectal Pejin et al. (2014) most likely by inducing apoptosis (Sakthivel et al., 2018).

The effect of the Makahiya (*Mimosa Pudica L.*) aqueous extract exhibited anti-angiogenic properties due to the significant reduction in branch points (Masagca, 2017). Additionally, the study results showed that Talisai (*Terminalia Catappa L.*) leaf extract has anti-angiogenic activity against CAM in chicken embryos and concluded that when the concentration of Talisai leaf extract increases, the reduction of small blood vessels also increases (Lontoc et al., 2012).

One-way ANOVA was used to examine the number of CAM vascularizations at three different concentrations (0.3 ml, 0.4 ml, and 0.5 ml) of *G. Procumbens* leaf extract (Table 3). The results showed a significant difference under conditions ($F(2,42) = 5.47$, p-value < 0.01). This finding suggested that the amount of *G. Procumbens* leaf extract substantially impacted CAM vascularization in chicken embryos with cancer-induced induction.

Table 3. Difference in the number of CAM vascularization among three G. procumbens leaf extracts

Amounts	No. of New Blood Vessels (Mean ± SD)	F (2,42)
Amount 1	26.60 ± 2.29	
Amount 2	24.87 ± 2.13	5.47 **
Amount 3	23.73 ± 2.71	
(-) Control	14.00 ± 1.94	
(+) Control	18.67 ± 1.29	

Legend: Amount 1 (0.3 ml), Amount 2 (0.4 ml), and Amount 3 (0.5 ml)
*** - Test is significant @ p-value<0.01; * - Test is significant @ p-value<0.05*

The post hoc analysis revealed that Amount 2 had a significant impact, as indicated by a mean vascularization count of 24.87 ± 2.13. The leaf extracts Amounts 1 and 3 (0.3 ml and 0.5 ml), with an average vascularization of 26.60 ± 2.29 and 23.73 ± 2.71 (Table 3), were not highly effective in inhibiting angiogenesis. In contrast, Amount 2 (0.4 ml), with an average vascularization of 24.87 ± 2.13, exhibited significant antiangiogenic activity, indicating its potential effectiveness in suppressing the formation of new blood vessels. The variation highlighted the significance of optimizing the amount of *G. Procumbens* leaf extract for therapeutic purposes focusing on decreasing angiogenesis in cancer treatment. This highlights the necessity for further investigation into its mechanisms and optimal dosage.

Angiogenesis is crucial for the uncontrollable growth and spread of cancer. Tumors can induce blood supply formation by releasing chemical signals that promote angiogenesis. They can also prompt nearby normal cells to generate molecules that signal for angiogenesis. These newly formed blood vessels provide the growing tumors with oxygen and other nutrients, enabling the cancer cells to invade adjacent tissues, spread to the body, and establish new cancer cell colonies known as metastases.

G. Procumbens is an effective chemotherapeutic agent against various cancer cell types with antiproliferative activity (Nisa et al., 2012; Maw et al., 2011). The anticancer effects of gamma-immunotherapy are exerted by influencing multiple stages of cancer development, such as initiation, cell growth, metastasis, and angiogenesis (Agustina et al., 2006). Blocking angiogenic pathways has been shown to inhibit tumor cell growth and angiogenesis (Hamid et al., 2013). *G. Procumbens* demonstrated antiangiogenic properties by preventing the formation of new blood vessels in chicken eggs (Hamid et al., 2013). The bioassays demonstrate that the leaves of this plant possess significant antioxidant, cytotoxic, thrombolytic, and antidiabetic properties. Hence, considering its potential therapeutic capabilities, it is imperative to thoroughly investigate and evaluate the plant to determine its intrinsic effectiveness and validate its medication use (Jobaer et al., 2023). The mechanism of action of this inhibitory effect has not been confirmed because G. procumbens leaf extract contains various phytochemicals, including flavonoids, saponins, tannins, terpenoids, and stroll glycosides (Puangpronp et al., 2010).

Benzene exposure can cause hematotoxicity and leukemia by targeting hematopoietic stem cells (HSCs), causing cancer and, in some cases, hematotoxicity. It is also considered an environmental pollutant and occupational toxicant (Zhu et al., 2013). Average increase in CAM collaterals following treatments with benzene induced showed leukemic activity in chicken embryos.

The benzene metabolite Roxarsone stimulates poultry and livestock products. Roxarsone can induce angiogenesis in mice Wang et al. (2013) through tube formation of vascular endothelial cells and growth. Benzene has a biphasic effect on angiogenesis. This effect is dose-dependent and affects angiogenic activity depending on the level of exposure. A low dosage of benzene yielded angiogenesis, whereas a higher concentration yielded an antiangiogenic effect. Roxarsone promotes angiogenesis in different human endothelial cells better than arsenic (ASIII) (Basu et al., 2008). Moringa oleifera ethanol extract effectively treats benzene-induced leukemia and is a natural anti-cancer agent (Akanni et al., 2014).

Overall, the data indicated that *G. Procumbens* leaf extract exhibited encouraging antiangiogenic properties, with different treatments demonstrating variable levels of effectiveness.

CONCLUSIONS

G. Procumbens leaf extract exhibited significant anti-angiogenic activity. This study showed that the extract inhibited new blood vessel growth in CAM induced by benzene. The findings demonstrated that *G. Procumbens* leaf extract affected blood vessel development in chicken embryos.

Based on the results of this study, several suggestions can be proposed.

1. Educational institutions may incorporate the findings of this research to promote interdisciplinary learning and practical scientific investigation.
2. The DOH and the FDA may support additional research and clinical trials to verify the effectiveness and safety of *G. Procumbens* as an anticancer agent.
3. Herbalists, pharmaceutical companies, and nutritionists are advised to consider *G. Procumbens* as a suitable supplementary treatment option supported by scientific evidence.
4. Future studies should examine some details regarding the mode of action of angiogenesis and its enduring impacts on tumor growth and metastasis.

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