Qualitative Screening and Analysis of Lunasia amara Blanco (Tawal-ulad) Ethanolic Leaves Extract as Potential Anti-Angiogenic Inhibitors Using the Chorioallantoic Membrane Assay on Mallard Duck Embryo

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ABSTRACT

Angiogenesis, the physiological process of forming new blood vessels, is a hallmark of tumor progression and metastasis in various cancers. As resistance to traditional chemotherapy increases, anti-angiogenic therapies have emerged as promising alternatives. This study investigates the anti-angiogenic potential of Lunasia amara Blanco (Tawal-ulad) ethanolic leaf extract using the chorioallantoic membrane (CAM) assay on mallard duck embryos. A quasi-experimental pretest-posttest design with non-equivalent groups was employed to assess the extracts inhibitory effect on vascular development across five treatment groups (1 mg/mL, 3 mg/mL, 5 mg/mL, pure extract) and two controls (positive: 1 mg/mL Celecoxib; negative: untreated). Results indicated that the pure extract yielded the highest average inhibition (68.89%), particularly in tertiary, quaternary, and quinary blood vessels, suggesting a concentration-dependent inhibitory trend. Despite these observable trends, one-way ANOVA and Tukey's post hoc tests revealed no statistically significant differences ($p > 0.01$) among treatments, indicating the effects may be due to biological variability. Nonetheless, the pronounced inhibitory patterns in higher extract concentrations support the need for further studies. These findings suggest Lunasia amara holds potential as a natural anti-angiogenic agent pending further validation through advanced assays and larger sample sizes.

Keywords: Angiogenesis, Anti-angiogenic, Lunasia amara Blanco, Chorioallantoic Membrane Assay, Celecoxib

INTRODUCTION

The global burden of angiogenesis-related disorders, particularly cancer, poses a significant health challenge, with over 9.6 million deaths annually attributed to cancer worldwide (WHO, 2024). Angiogenesis, the formation of new blood vessels, plays a crucial role in tumor progression by supplying nutrients and oxygen to cancerous cells. In the Philippines, cancer remains a leading cause of mortality, contributing to approximately 66,000 deaths annually (Calimag and Silbermann, 2019). According to Wang et al. (2021), a major issue in cancer treatment is the development of resistance to chemotherapeutic drugs, often associated with abnormal blood vessels within the tumor microenvironment. Jain's vascular normalization theory, introduced in 2001, suggests that remodeling the structure and function of these abnormal vessels through angiogenesis inhibitors can enhance the efficacy of chemotherapeutic drugs. This theory underscores the critical role of anti-angiogenic therapies not only in disrupting new blood vessel formation but also in normalizing existing vessels to optimize drug delivery.

Angiogenesis cascades in cancers; the angiogenesis transition was initially thought to be caused by the ectopic synthesis and production of growth factors for tumor cells. The sprouting of new blood vessels lies at the heart of wound healing, delivering essential oxygen and nutrients for tissue repair. Impaired angiogenesis significantly hinders this process, perpetuating chronic wounds. Numerous studies have elucidated the crucial role of vascular endothelial growth factor (VEGF) and its receptors in orchestrating this intricate vascular dance (Shibuya, 2011; Sholley et al., 2019).

Modern research and experiments have been conducted in order to figure out synthetic ways to inhibit angiogenesis, one of which is the Anti-angiogenic Therapy. According to some scientists, it is less susceptible to the development of treatment resistance because of its direction toward the stroma rather than to the tumor cells (Li, Kang, Wang, & Huang, 2018).

According to Ean-Jeong Seo (2013), over 50,000 plants possess therapeutic virtues, and about 80% of the world population uses herbal medicines to combat angiogenesis-related disorders. Moreover, Tawal-ulad is one of the promising traditional medicinal plant sources with anti-angiogenic properties. Tawal-ulad extraction treatments caused the inhibition of branches of blood vessels and suppression of the branching points of primary, secondary, and tertiary blood vessels, thus demonstrating potential anti-angiogenic activity. However, the angiogenic activity of the Tawal-ulad (Lunasia amara Blanco) leaves has yet to be fully explored.

In this study, the researchers investigated the anti-angiogenic potential of Tawal-ulad leaves, which require validation in vitro using the Chorioallantoic Membrane (CAM) assay. By understanding its inhibitory effects on blood vessel formation and linking these effects to vascular normalization, this research seeks to develop cost-effective therapeutic approaches for combating angiogenesis-related disorders. Moreover, the findings could contribute to addressing the limitations of existing treatments, such as drug resistance and high toxicity, by exploring plant-based solutions.

MATERIALS AND METHODS

Plant Collections and Authentication

Two hundred (200) grams of fresh and disease-free mature leaves of the Lunasia amara Blanco (Tawal-ulad) were collected in Barangay Cannery Site, Polomolok, South Cotabato, which is located between General Santos City and Tupi.

The plant authentication process for the Lunasia amara Blanco (Tawal-ulad) leaf was in Cotabato. This process ensures that the samples are collected at the University of Southern Mindanao, Kabacan, Cotabato, providing accurate identification and verification of the species before proceeding with the analysis.

Preparation of plant extracts

The collected Lunasia amara Blanco (Tawal-ulad) leaves were washed thoroughly with distilled water and air-dried for 72 hours. Dried leaves were pulverized using a blender and stored in a resealable bag (Ziploc) at room temperature until further use. The crushed dried leaves of 30 grams were completely soaked in 300 mL of absolute ethanol for 48 hours. The resulting extract was filtered using filter paper. The concentrated filtrates were transferred to an evaporating dish and evaporated using a rotary evaporator at a constant temperature of 40ºC to 60ºC to incubate the samples over an extended period (Gamallo et al., 2016).

CAM Assay Preparation

The CAM Assay was performed to evaluate the anti-angiogenic activity of Lunasia amara Blanco (Tawal-ulad) ethanolic leaf extract. The experimental methods used in the study were adapted from Gamallo et al.'s (2016) study.

Eighteen mallard duck eggs that were four days old and weighed at least 50 g were obtained from a local hatchery in Tantangan, South Cotabato. Candling tests were conducted before experimentation to verify the viability of the embryos. A spotlight was positioned on the side to view the location of the growing embryo. Eggs with underdeveloped or dead embryos were discarded.

The application of 70% ethanol was sprayed on each egg to minimize the chance of inflammation from the outer covering of the egg. The eggs were dried out with air pressure and incubated at a temperature of 37 °C with a moisture level of 65.5 % for 72 hours (three days). A perforation of the window, about 2 cm in width, was performed using a mini electric grinder. Using a syringe of 4-5 mL syringe, white egg (albumen) was

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removed. The windows were sealed with parafilm, and the duck eggs were returned to the incubator.

After a three-day incubation, the windows of every duck egg were opened using a mini electric grinder to administer the different dilutions simultaneously in each respective subgroup of growing CAMs: 1 mg/mL, 3 mg/mL, 5 mg/mL, and pure extract. A blank control was used as a negative control, and 1 mg/mL Celecoxib served as a positive control. Approximately 100 µL of the controls (positive and blank) and working solutions (1, 3, 5 mg/mL, and pure extract) were applied to the CAM using a micropipette. The administration of the different treatment doses, including the Lunasia amara Blanco (Tawal-ulad) ethanolic leaf extracts, the positive control, and the negative control, was conducted in triplicate to ensure the reliability and reproducibility of the experimental results. The windows were tightly sealed again with parafilm and placed back into the incubator for one day (24 hours). After 24 hours, the sealed windows were unsealed using a photo stereomicroscope, and images of the shape and zone of the CAM were captured and examined (Gamallo et al., 2016).

Visual Assessment and Photography

The researcher carefully examine the photos using the photo stereomicroscope, that were taken for the number of new blood vessel branch points that grew or sprouted from the major blood vessels and counted systematically in a clockwise manner from the right hemisphere to the left hemisphere (Gamallo et al., 2016), and noted the changes in blood vessels (Raju & Ying, 2023).

Data Analysis

The anti-angiogenic activity of the treatments was expressed as Mean ± S.E.M using one-way ANOVA and pairwise comparisons among the treatments using Tukey's post-hoc test at $p < 0.01$. The statistical analysis was conducted using SPSS 17.0. One-way ANOVA is a statistical method utilized for testing differences in the means of three or more groups (One-Way ANOVA, n.d.

RESULTS AND DISCUSSION

Table 1: Percentage Inhibition of the Anti-Angiogenic Activity of Lunasia amara Blanco (Tawal-ulad) Leaf Extract Assessed via CAM Assay across the Branching levels of blood vessels through the different concentrations

Branching levels of blood vessels	Negative Control (%)	1 mg/mL (%)	3 mg/mL (%)	5 mg/mL (%)	Pure Extract (%)
Primary	0.00%	0.00%	40.12%	0.00%	0.00%
Secondary	19.26%	22.33%	45.13%	52.43%	30.03%
Tertiary	17.80%	-7.44%	39.98%	10.34%	77.78%
Quaternary	-54.13%	-9.27%	-8.38%	43.30%	83.33%
Quinary	—	2.17%	-85.65%	78.99%	95.32%
Average Inhibition	26.51%	0.77%	12.64%	41.18%	68.89%

Table 1 presents the percentage of anti-angiogenic activity exhibited by Lunasia amara Blanco (Tawal-ulad) leaf ethanolic extract using the chorioallantoic membrane (CAM) assay across the branching levels of blood vessels through the different concentrations. Results indicate varying levels of blood vessel inhibition depending on both the branching levels and the concentration of the extract.

In the the primary blood vessels, no inhibitory effect was observed in any group except at 3 mg/mL, which showed a notable 40.12% inhibition. During the secondary blood vessels, the extract displayed increasing anti-

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angiogenic effects with concentration: 22.33% at 1 mg/mL, 45.13% at 3 mg/mL, 52.43% at 5 mg/mL, 30.03% at pure extract, and 19.26% in the negative control.

In the tertiary blood vessels, the pure extract demonstrated strong inhibitory activity (77.78%), followed by 3 mg/mL (39.98%). However, 1 mg/mL showed a slight negative effect (-7.44%), suggesting limited efficacy at lower concentrations. For the quarternary blood vessels, the pure extract again showed high inhibition (83.33%) along with 5 mg/mL (43.30%), while the lower concentrations presented negative values, indicating no suppression of angiogenesis.

At the quinary blood vessels, the pure extract (95.32%) and 5 mg/mL (78.99%) concentrations yielded the highest inhibition rates. The 1 mg/mL showed only 2.17%, with 3 mg/mL even reflecting a strong negative value (-85.65%).

When averaged across the branching levels of blood vessels, the pure extract consistently demonstrated the highest inhibition (68.89%), followed by 5 mg/mL (41.18%) and 3 mg/mL (12.64%). The lower concentration 1 mg/mL exhibited minimal average inhibition (0.77%).

These results suggest that higher concentrations of Lunasia amara Blanco (Tawal-ulad) extract, particularly the pure form, possess strong anti-angiogenic potential, most notably during the later stages of blood vessel development. The pronounced effect of the Pure Extract may be attributed to its higher concentration of secondary metabolites such as flavonoids, alkaloids, tannins, and phenols, which are known to exhibit anti-proliferative and anti-angiogenic properties (Putri and Tawali, 2024). Flavonoids within the extract can modulate angiogenic pathways by inhibiting VEGF signaling and endothelial cell migration—key processes in neovascularization (Kim et al., 2016

Table 2: Percentage Inhibition of Positive Control (1mg/mL Celecoxib) across the Branching levels of blood vessels

Branching levels	**Positive Control (%)**
Primary	0.00%
Secondary	-50.00%
Tertiary	21.18%
Quaternary	-11.32%
Quinary	18.75%
Average Inhibition	7.52%

Celecoxib has been shown to significantly reduce angiogenesis in the chick embryonic chorioallantoic membrane (CAM) assay. Furthermore, it inhibits both the growth and microvascular density of the murine TA3-MTXR tumor. In addition, Celecoxib decreases the microvascular density of tumor metastases. Moreover, it promotes apoptosis while simultaneously reducing vascular endothelial growth factor (VEGF) production and suppressing cell proliferation within the tumor (Rosas et al., 2014). The anti-angiogenic and antitumor effects of Celecoxib appear to align with its previously observed activity on other tumor cell lines, indicating a possible involvement of prostaglandins (PGs) and VEGF production. Consequently, these findings suggest the potential for combining Celecoxib with other experimental therapies. Ideally, such combinations could lead to synergistic effects, enhancing overall treatment efficacy.

In our results, Table 2 presents the percentage inhibition of the Positive Control (1 mg/mL Celecoxib) across different branching levels of blood vessels. At the secondary blood vessel level, the positive control resulted in a negative inhibition value (-50.00%), indicating a possible pro-angiogenic or inconsistent effect. At tertiary blood vessels, it showed 21.18% inhibition, while at quinary blood vessels, it showed 18.75%. The positive control averaged only 7.52%, possibly due to natural variation. These results indicate that Celecoxib exhibited mild anti-angiogenic activity, primarily affecting tertiary and quinary vessel levels. In contrast, it showed no inhibition at the primary vessel level and even stimulated vessel formation at the secondary and quaternary levels, as reflected by the negative inhibition values. This suggests that while Celecoxib can inhibit the proliferation of finer capillary networks, its overall activity may vary depending on vessel type and branching level. The average inhibition of 7.52% across all branching levels implies a relatively low anti-angiogenic effect under the conditions tested.

The disparity in the anti-angiogenic effect of Celecoxib as our positive control may be due to the choice of solvent. In this study, Celecoxib was dissolved in water, whereas it is known to be soluble in organic solvents such as ethanol and DMSO, which maximize the effect of the stock solution (Cayman Chemical, 2022). According to Nowak-Sliwinska et al. (2018), consensus guidelines and multiple CAM-method reviews emphasize that the choice of carrier/vehicle (filter paper, rings, solvents, surfactants), as well as irritation from the carrier or test material, may cause inflammation or vessel changes that mask or mimic angiogenic/anti-angiogenic activity.

In conclusion, Celecoxib may possess an anti-angiogenic mechanism that is more effective in later-stage, fine vessel formation, rather than in the inhibition of major vascular structures. However, under the conditions tested in this research, it demonstrated a relatively low anti-angiogenic effect.

Table 3. One-way ANOVA Summary for Treated Groups (Total Vessel Counts)

Source of Variation	**Sum of Squares (SS)**	**Degrees of Freedom (df)**	**Mean Square (MS)**	**F-value**	**p-value**	**F critical (F crit)**
Between Groups	208.71	5	41.74	1.46	0.2402	3.8951
Within Groups	687.08	24	28.63			
Total	895.78	29				

Table 3 presents the one-way ANOVA summary analyzing the total vessel counts across the treated groups. The between-groups variation has a sum of squares (SS) of 208.71 with 5 degrees of freedom, resulting in a mean square (MS) of 41.74. The within-groups variation shows a larger sum of squares of 687.08 with 24 degrees of freedom and a mean square of 28.63. The calculated F-value is 1.46, which is lower than the critical F-value of 3.8951, and the corresponding p-value is 0.2402. Since the p-value exceeds the significance level of 0.01, the analysis indicates that there is no statistically significant difference in total vessel counts among the different treatment of Tawal-ulad (Lunasia amara Blanco) leaf extract, suggesting that either the dosage or experimental conditions were insufficient to demonstrate a meaningful inhibitory effect. However, it shows minimal anti-angiogenic effects showing potential statistically as shown by the inhibition of blood vessels.

Table 4. Pairwise Comparisons of Anti-Angiogenic Effects Using Tukey's HSD Post Hoc Test ($\alpha = 0.01$) at the different concentrations (1 mg/mL, 3 mg/mL, 5 mg/mL, and the pure extract)

Comparison	**Mean Difference**	**p-value (0.01)**	**Interpretation**
Pure Extract vs Tawal-ulad 1 mg/mL	7.134	0.3165	No significant difference
Pure Extract vs Tawal-ulad 3 mg/mL	3.536	0.8978	No significant difference
Pure Extract vs Tawal-ulad 5 mg/mL	1.534	0.9973	No significant difference

Tawal-ulad 1 mg/mL vs 3 mg/mL	-3.598	0.891	No significant difference
Tawal-ulad 1 mg/mL vs 5 mg/mL	-5.6	0.5725	No significant difference
Tawal-ulad 3 mg/mL vs 5 mg/mL	-2.002	0.9906	No significant difference

Table 4 it presents the anti-angiogenic activity of Lunasia amara Blanco (Tawal-ulad) ethanolic leaf extract compared across the different concentrations: 1 mg/mL, 3 mg/mL, 5 mg/mL, and the pure extract. Pairwise comparisons were performed using Tukey's HSD post hoc test at a significance level of $\alpha = 0.01$. The results showed **no statistically significant differences** in anti-angiogenic activity among any of the concentrations. All p-values observed in the data that were well above the threshold of 0.01**,** indicating that the differences in anti-angiogenic activity between concentrations were not statistically significant. This suggests that increasing the concentration did not produce a statistically stronger inhibition of blood vessel formation within the tested range.

Extraction is widely recognized as a critical step in achieving high purity and maximum recovery of specific substances from plants. However, it often presents challenges, particularly when isolating a compound present in low concentrations within complex plant matrices. This difficulty arises because major components typically dominate the extract in large proportions. Therefore, several factors—such as the extraction method, particle size, type of solvent used, properties of the target compound, and the presence of impurities—play a vital role in determining the overall efficiency and success of the extraction process, Do et al. (2014). In addition, extraction is acknowledged as the most important part of obtaining high purity and recovery of a certain substance from plants. It is also very challenging to extract a fairly limited compound from plant matrixes because the major components are mostly present in the extract with a high percentage

Table 5. Pairwise Comparisons of Anti-Angiogenic Effects Using Tukey's HSD Post Hoc Test ($\alpha = 0.01$) at the negative control and positive control (1mg/mL Celecoxib)

Comparison	Mean Difference	Adjusted p-value	Interpretation
Celecoxib vs Negative Control	-0.736	0.9999	No significant difference
Celecoxib vs Pure Extract	-6.536	0.4085	No significant difference
Celecoxib vs Tawal-ulad 1 mg/mL	0.598	1.0	No significant difference
Celecoxib vs Tawal-ulad 3 mg/mL	-3.0	0.9461	No significant difference
Celecoxib vs Tawal-ulad 5 mg/mL	-5.002	0.6807	No significant difference
Negative Control vs Pure Extract	-5.8	0.5363	No significant difference
Negative Control vs Tawal-ulad 1 mg/mL	1.334	0.9986	No significant difference
Negative Control vs Tawal-ulad 3 mg/mL	-2.264	0.9837	No significant difference
Negative Control vs	-4.266	0.8027	No significant difference

Tawal-ulad 5 mg/mL			

Table 5 shows the results of pairwise comparisons of the anti-angiogenic effects of different treatments using Tukey's HSD post hoc test with a significance level of $\alpha = 0.01$. Across comparisons—including Celecoxib versus the negative control—none of the mean differences were statistically significant, as indicated by adjusted p-values all well above the 0.01 threshold. This suggests that no treatment group differed significantly from any other in terms of inhibiting angiogenesis under the conditions tested. Even comparisons between the negative control and treatment groups failed to reach significance, indicating that the observed differences could be due to random variation rather than true treatment effects. Although Celecoxib and higher concentrations of Tawal-ulad extract showed trends of inhibition, these did not reach statistical significance in pairwise comparisons, indicating that observed differences may be due to variability rather than definitive treatment effects.

In recent years, a growing number of bioactive plant compounds have been investigated for their antiangiogenic properties. Notably, flavonoids have emerged as some of the most extensively studied. These compounds exert their antiangiogenic and antimetastatic effects by modulating various signaling pathways. Specifically, flavonoids influence the expression of vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and epidermal growth factor receptor (EGFR), while also inhibiting key pathways such as NF-κB, PI3K/Akt, and ERK1/2. As a result, they produce potent antiangiogenic effects. Accordingly, this review seeks to present the latest insights into the molecular mechanisms underlying the antiangiogenic actions of natural flavonoids. Subbaraj et al. (2021)

CONCLUSION

In conclusion, while Tawal-ulad extract at higher concentrations, particularly the pure extract**,** demonstrated promising anti-angiogenic effects, particularly in later-stage vessels, the results did not reach statistical significance when compared to Celecoxib or the untreated control under stringent conditions. Given the limitied studies on Tawal-ulad, this work provides preliminary evidence of its potential as an anti-angiogenic agent. This study suggests that Lunasia amara has the potential to be developed as an anti-angiogenic agent**,** but further investigations with larger sample sizes and additional experimental methods are needed to validate these findings and determine the full therapeutic potential of Tawal-ulad as a candidate for angiogenesis-related diseases.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

All authors have declared no conflict of interest.

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