Evaluation of Anti-angiogenic Activity of the Acetone Leaf Extract of *Annona squamosa* Linn. (Annonaceae)

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ABSTRACT

Objective. The study aimed to investigate the potential anti-angiogenic effects of acetone extracts from *Annona squamosa* leaves *in vivo*.

Methods. Crude acetone extract of *A. squamosa* leaves was prepared via simple maceration. Physicochemical and phytochemical screening of the extract were also performed. The anti-angiogenic effect of *A. squamosa* was assessed using *in vivo* chorioallantoic membrane (CAM) assay, with quantitative analysis performed using ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA). The eggs were treated with 1000 ppm, 500 ppm, and 250 ppm doses of the extract. Quercetin was utilized as the positive control while distilled water was used as the negative control.

Results. The results of the study showed the presence of alkaloids, flavonoids, tannins, and phenolic compounds in the leaves of *A. squamosa*. CAM assay revealed a significant (P <0.001) inhibition of blood vessel growth after 48 hours of treatment with the crude acetone extracts, particularly at the lowest concentration (250 ppm). Statistical analysis confirmed the similarity in anti-angiogenic effect between the *A. squamosa* extract and Quercetin.

Conclusion. The acetone extract of *A. squamosa* leaves possesses anti-angiogenic properties *in vivo*, suggesting its potential for developing anti-angiogenic agents. Further research to identify and isolate the specific bioactive compounds responsible for this activity is recommended.

Keywords: angiogenesis, Annona squamosa, CAM Assay

* *Ms. Loretizo and Ms. Alegado-Bagaoisan shared first authorship for this manuscript.*

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INTRODUCTION

Angiogenesis, from the Greek word *Angêion*, meaning "vessel formation", refers to the emergence of blood vessels from already existing vasculature. It is a metabolic activity controlled by various biomolecules and chemical signals in the body that normally occurs throughout life in health and even in diseases.[1] Moreover, angiogenesis is an essential process for body development, wound healing, and growth. However, in cases where there is an imbalance in chemical signals to control angiogenesis, it can result in the uncontrolled rise in blood vessels which causes developing tumours to

enlarge, and cancer cells to invade neighbouring tissues or spread throughout the body and metastasize.[2] Consequently, improper blood vessel formation may lead to exacerbation of pathogenesis, such as cancer, diabetes, arthritis, coronary artery disease, and a variety of other conditions.[3]

Recent statistics show that in 2020, cancer was the cause of around 10 million deaths globally, that was roughly one in six fatalities.[4,5] Neoplasms, also known as cancer, accounted for 3,881 cases or 8.9% of the deaths in the Philippines, making it the third leading cause as of March 2022. In the year 2021, among the leading causes of death in the country was cancer as well.[6]

Angiogenesis may be a key factor in the initial development of cancer.[7,8] During carcinogenesis, angiogenesis is one of the main factors for tumour advancement and metastasis.[9] In addition, tumour growth requires a high metabolic rate wherein a constant supply of nutrients is required, which can be achieved through angiogenesis.[10] Moreover, angiogenesis is also associated with inflammatory processes linked to tumour progression.[11]

Annona squamosa Linn., also known as custard apple, sugar apple, sweet apres, sitaphal, sweet sop, and locally known as *atis,* is an evergreen plant that is part of the Annonaceae family. *A. squamosa*, aside from its role in photosynthesis, is deemed to be a potential source of natural drug products. The phytochemicals that were found to be most prevalent in the plant's leaves were saponins, flavonoids, alkaloids, phenols, total phenolic compounds (TPCs), and tannins.[12] Additionally, *A. squamosa* is known to have a wide range of ethnopharmacological properties that can be useful for the treatment of different illnesses.[13] As stated in a study of Ayurveda, the fruit of *A. squamosa* can enrich the blood and increase muscle strength due to its tonic effect.[14] In traditional Chinese medicine, *A. squamosa* seeds were a good remedy for malignant wounds as a result of cancer.[14] Moreover, a group of researchers from the University of the Philippines Los Baños concluded that *A. squamosa* contains total phenolic content and total flavonoids that contribute to its antioxidant activity.[15]

The bioactive compounds of a plant play a significant role in both medicinal and agricultural essence. Chemical compounds such as diterpenes (DITs), cyclopeptides, and annonaceous acetogenins (ACGs) were considered as the main components found in *A. squamosa*. Previous studies showed the proliferative effects of ethanol-extracted *A. squamosa* seeds on human cancer cell lines, namely human embryonic kidney cells (HEK 293), breast cancer cell lines (MCF7), and liver cancer cells (HepG2) using the MTT assay. It was reported that *A. squamosa* seeds can inhibit the proliferation of MCF7 cells due to the bioactive compounds that these extracts contain.[16] This correlates to the findings of another study that discovered *A. squamosa* leaf isolate had cervical anticancer activity at a sample concentration of 70.9021 ppm using MTT assay.[17] Further, various *in vivo* and in *vitro* studies show a more defined mechanism through apoptosis of cancer cells induced by annonaceous acetogenins (ACGs) extracted from the seeds of *A. squamosa*.[18]

Several studies have been conducted to investigate the possible bioactivities of *A. squamosa* such as antidiabetic, antimalarial, antiparasitic, and anticancer properties. While there are existing investigations on the bioactivities of *A. squamosa*, there is relatively little data that shows the activities of the leaves against angiogenic-dependent diseases. The present study is designed to evaluate the anti-angiogenic role of the crude extract of *A. squamosa* leaves *in vivo* using the chorioallantoic membrane (CAM) assay.

METHODS

Study Design

This study utilized an experimental research design with a quantitative approach, involving intentional interventions and measurement of outcomes.[19] The study was carried out at the Biology Laboratory in the Pharmacy Department of Adamson University, thereby ensuring high internal validity but potentially limiting external validity. Data collection involved experimentation to test the research hypothesis, with careful consideration given to selecting an appropriate design for this purpose. Figure 1 shows the flow chart of the experimental methods used in this study.

Plant Collection and Identification

Approximately 5 kilograms of *A. squamosa* leaves were gathered from Maragondon, Cavite, Philippines. The leaves were thoroughly rinsed with tap water and distilled water to eliminate any debris and subsequently air-dried in the shade at room temperature for 72 hours.[20] The identity of the plant was confirmed through authentication at the Jose Vera Santos Memorial Herbarium, Institute of Biology, College of Science, University of the Philippines-Diliman, certifying that the leaves used in the study were from *A. squamosa* plants cultivated in the Philippines.

Plant Extraction

Dried leaves of *A. squamosa* were ground into a coarse powder and then macerated with acetone in a 1:10 ratio (weight in volume) in a sealed container and agitated periodically for three days until all soluble components were dissolved.[21,22] After filtering the resultant extract, a rotary evaporator was used to concentrate the filtrate (crude acetone extract). The crude extract was further evaporated under a fume hood at room temperature overnight. It was then stored in a light-protected sterile container at 2-8 °C until use. The percentage extract yield was calculated using the formula:

$$\text{Extraction yield (\%)} = \frac{\text{weight of the crude extract}}{\text{weight of the dried plant sample}} \times 100$$

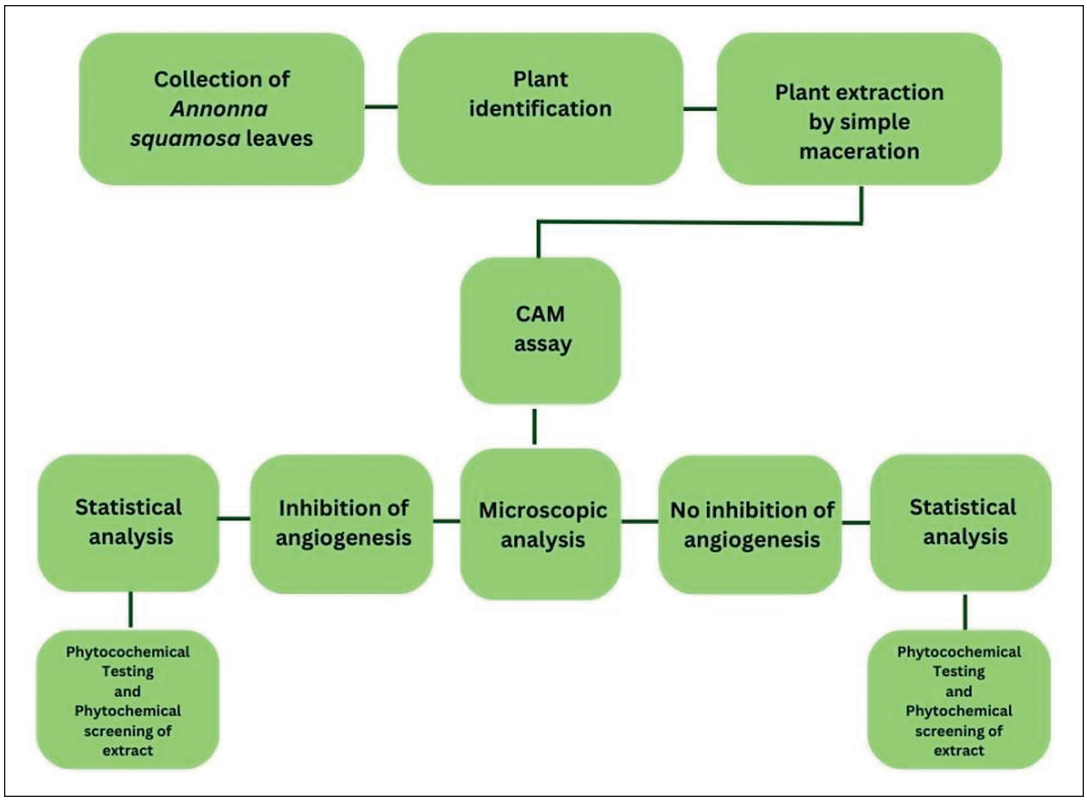

Figure 1. The experimental flow diagram for the extraction of *A. squamosa*, from collection of the leaves to the CAM assay, and its analysis.

Sample preparation

A. squamosa extract was dissolved in acetone to create a stock solution and produce serial dilutions at concentrations of 1000, 500, and 250 ppm. Quercetin was initially dissolved in dimethylsulfoxide (DMSO) and prepared at a concentration of 1 mg/mL, whereas distilled water was used as negative control. The prepared samples were stored at 2-8°C until further analysis.[23,24]

Physicochemical Parameters of the Extract

Organoleptic Properties

The organoleptic properties of the acetone extract of *A. squamosa* leaves evaluated include its color and odor.

Determination of pH

The pH of acetone extract of *A. squamosa* leaves were determined using a basic laboratory pH meter (Aquasearcher™ AB33PH Bench meter). The pH was assessed while the electrodes were submerged in the solution at room temperature.

Solubility

The acetone extract of *A. squamosa* leaves was dissolved in water, chloroform, ethanol, and diethyl ether to assess the solubility.

Phytochemical Screening of the Extract

Chemical constituents including alkaloids, tannins, saponins, flavonoids, glycosides, and phenolic compounds were analyzed in the acetone extract of *A. squamosa* leaves whether they were present or absent by following standard methods.[25-27] Depending on how the test turned out, the result was either marked as present (+) or absent (-). Every experiment was performed in triplicates.

The presence of alkaloids was detected using Mayer's test; flavonoids using Mg turning test; tannins using Braymer's test; saponins using Foam test; glycosides using Keller Kiliani's test; and phenolic compounds using Ferric chloride test.

Chorioallantoic Membrane (CAM) Assay

The study employed the chorioallantoic membrane (CAM) assay to assess the angiogenic potential of the crude acetone extract from *A. squamosa* leaves. Five-day-old fertilized duck eggs were procured from Ciriako Alvarez Duck Farm in Barangay Bundukan, Nasugbu, Batangas. With distilled water, the eggs were cleansed and then pre-incubated horizontally in an Isotherm® laboratory incubator at a constant temperature of 37.5°C and humidity. Distilled water was used for cleaning to avoid potential harm to the embryos that might be caused by commonly used disinfectants like ethanol.[28] On the 5th day of incubation,

egg candling was performed to check the embryo's position and assess egg viability. On the 7th day of incubation, the area where a small opening was made in each egg was sterilized with 70% ethanol. Approximately 3 mL of albumin was removed from each of the eggs using a syringe, and the hole was sealed with adhesive tape. This step facilitated better access and quantification of CAM vasculature by allowing the detachment of the membrane from the outer shell. Before cutting the viewing window, a layer of tape was applied to the eggshell to prevent any shell fragments from potentially contaminating or causing inflammation on the CAM.[29] A sterile fine-cutting tool was used to create a window of approximately 1 x 1 cm on the membrane, exposing the CAM. The window was covered with Parafilm before the eggs were placed back into the incubator. The filter discs were applied on day 11 of incubation, a stage when CAM vascularization is most rapid and responsive to anti-angiogenic factors.[30]

Filter paper discs, with a diameter of 5 mm, were used to carry the plant sample, quercetin (positive control), and distilled water (negative control).[31] A sterile metal hole puncher was used to create these discs, which were sanitized with 70% ethanol and subsequently air-dried beneath a laminar flow hood. The discs were loaded with 10 µL of the sample and allowed to air-dry before they are applied directly onto the CAM. After application, the eggshell opening was resealed with Parafilm, and the eggs were continued to be incubated at 37±1˚C until day 13. Upon reaching day 13, the treated CAMs were exposed to remove the filter discs for further observation and analysis of blood vessels.

The CAM assay was conducted in a biosafety cabinet (BSC) under the supervision of trained laboratory personnel to ensure adherence to biosafety protocols.

All equipment and surfaces were thoroughly sanitized with 70% alcohol before and after the procedures to minimize contamination risk.

Imaging and Quantification of CAM

The CAM assay utilized a stereomicroscope to observe and evaluate angiogenic activity.[32,33] Only healthy embryos with well-developed vascular structures and no signs of hemorrhage were included in the assessment. The eggs were carefully positioned on sterile Petri dishes, and the CAMs were examined and photographed under the stereomicroscope 48h after treatment. The resulting microphotographs were analyzed using *ImageJ* software (U. S. National Institutes of Health, Bethesda, Maryland, USA) which converted the images to grayscale and enabled the quantification of CAM vessels within the region where the filter paper discs were initially applied for each treatment group.

Statistical Analysis

The data was analyzed using Stata Statistical Software 14.2 (Stata Corporation, College Station, TX), and results were presented as means ± standard deviation (SD). Tukey's post-hoc analysis was utilized after one-way analysis of variance (ANOVA) to identify significant differences between the experimental and control groups. P-values <0.05 were used to determine statistical significance.

Ethical Considerations

The research was conducted with approval from the Biosafety Review Officer at Adamson University - College of Pharmacy (2023-AdU-04-PHA-01-003). Because the eggs are not thought to contain live animals until day 17 of development, the CAM assay does not require authorization from an ethics committee before the experiment is allowed to proceed.

RESULTS

Plant Extraction

The yield of *A. squamosa* leaves extract using acetone as solvent in the extraction process was found to be 10.6%.

Physicochemical Parameters of *A. squamosa* Leaves Extract

The acetone extract of *A. squamosa* leaves was dark green with a characteristic aroma. The pH of the solution is 5.93. It was sparingly soluble in water, slightly soluble in ethanol, and freely soluble in chloroform and diethyl ether.

Phytochemical Screening of the Extract

Utilizing a qualitative approach, the phytochemical components of *A. squamosa* leaves were identified. (Table 1). The acetone extract revealed the presence of flavonoids, phenolic compounds, alkaloids, and tannins. (Appendix)

Table 1. Results of the Phytochemical Analysis of the Acetone Extract of *A. squamosa* Linn. Leaves

Phytochemicals	Test	Expected Positive Test Result	Observation
Alkaloids	Mayer's test	yellow/creamy white precipitate	(+)
Falvonoids	Mg turning test	crimson red color	(+)
Tannins	Braymer's test	blue/green-black solution	(+)
Saponins	Foam test	formation of frothing	(-)
Glycosides	Keller Kiliani's test	formation of brown ring/bluish-green color	(-)
Phenolic compounds	Ferric chloride test	deep blue/black color	(+)

(+) presence, (-) absence

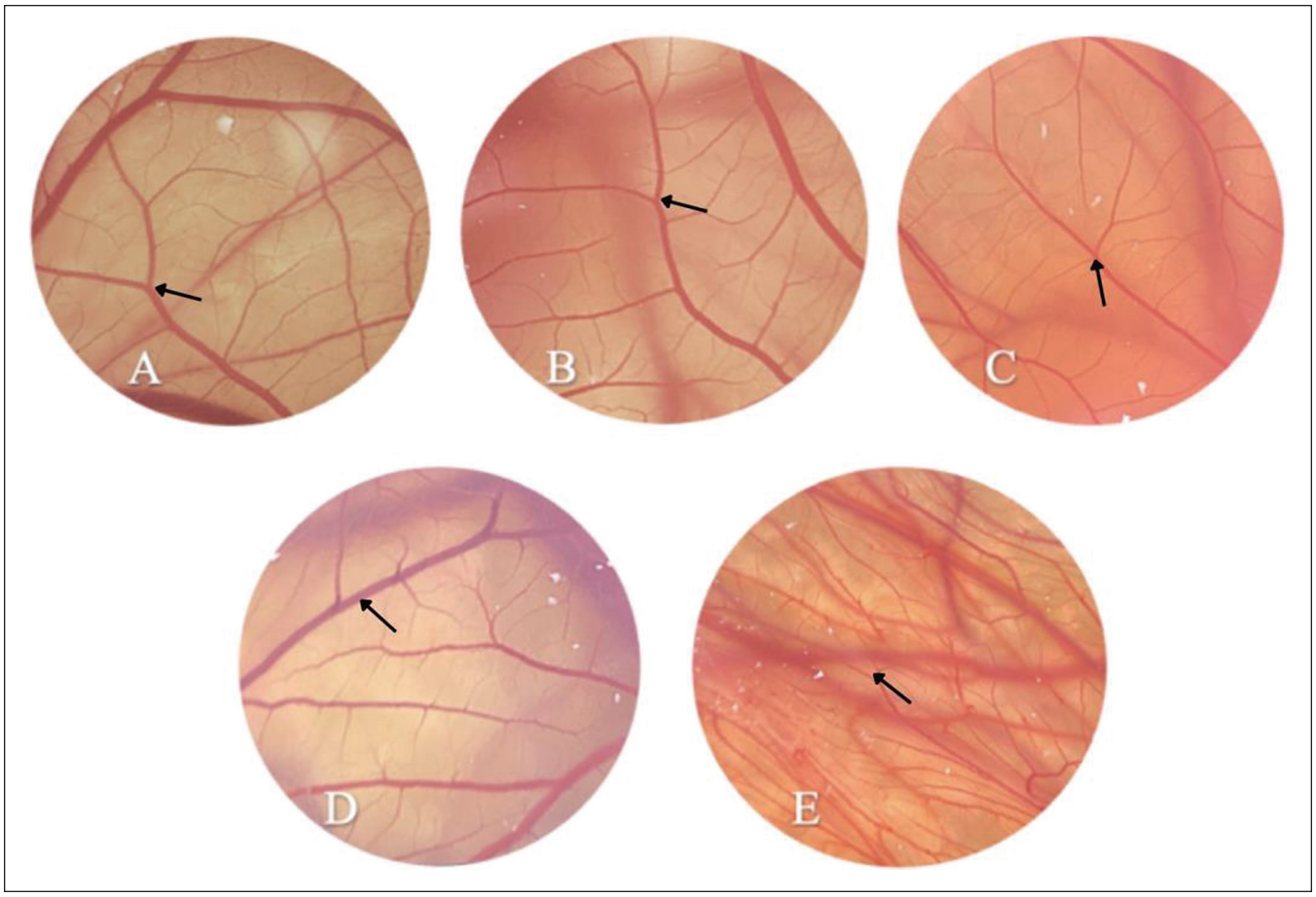

Figure 2. Representative images of CAMs treated with various concentrations of *A. squamosa* extract at 1000 ppm in **(A)**, 500 ppm in **(B)**, 250 ppm in **(C)**, while positive control (Quercetin) is shown in **(D)**, and negative control (distilled water) is represented in **(E)**. The major blood vessels where the growth begins are indicated by the arrows.

Chorioallantoic Membrane (CAM) Assay

The study assessed the anti-angiogenic properties of *A. squamosa* leaf extract using the CAM assay. Different concentrations of *A. squamosa* leaf extract (1000 ppm, 500 ppm, and 250 ppm) were tested alongside quercetin (positive control) and distilled water (negative control). Observation under a stereomicroscope revealed modest inhibition of blood vessel development in all groups. The highest concentration (1000 ppm) showed a more significant reduction in blood vessel formation compared to the group treated with 500 ppm. Group C, treated with the lowest concentration (250 ppm), displayed the fewest increase in blood vessels in the area where the disc was placed. The positive control group (quercetin) exhibited notable inhibition of blood vessel formation, while the negative control group showed normal vascular development with clear networks and prominent vessels, indicating healthy embryonic growth.

The results of the *in vivo* CAM assay revealed that the acetone extracts of *A. squamosa* leaves had a significant impact on the process of angiogenesis in the chorioallantoic membrane (CAM). Interestingly, by performing microscopic analysis, the concentration of 250 parts per million (ppm) of *A. squamosa* leaves extract exhibited the strongest inhibitory effect on the development of CAM blood vessels, as depicted in Figure 2.

The data presented in Figure 3, analyzed using *ImageJ* software, shows the percent change in number of blood vessels relative to the negative control. At a 1000 ppm dose, *A. squamosa* extract (Group A) shows moderate activity (-49.7% reduction). Group B, treated with 500 ppm, shows the least activity (-28.9% reduction), while Group C, treated with 250 ppm, shows the strongest anti-angiogenic activity (-85.7% reduction). Notably, Group C, with the lowest dose of 250 ppm, exhibited the most significant reduction in blood vessel count compared to groups receiving higher doses. Quercetin (positive control) shows -94.2% reduction, confirming its known anti-angiogenic properties.

A highly significant effect ($P < 0.001$) was shown by One-Way Analysis of Variance (ANOVA) on the angiogenesis of duck embryo CAM after the introduction of *A. squamosa* leaf extracts. The ANOVA analysis focused on the mean number of blood vessels in the chorioallantoic membrane when

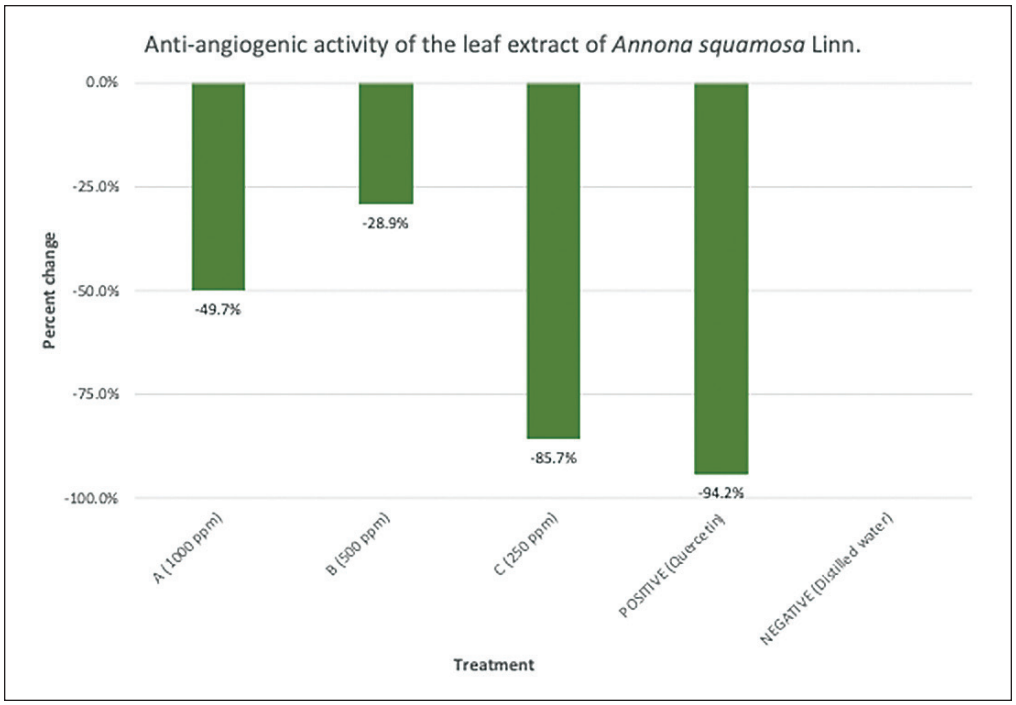

Figure 3. Graph of the percent change in number of blood vessels relative to negative control.

Note: Data are expressed as mean ± standard deviation of three independent experiments.

exposed to different concentrations of *A. squamosa*. Tukey's test confirmed and supported the results obtained from the ANOVA.

The comparisons between various treatments revealed significant differences, with p-values below 0.05, indicating variability in their activities. Specifically, groups treated with *A. squamosa* leaf extract at doses of 1000 ppm and 500 ppm, 1000 ppm and 250 ppm, and 1000 ppm and Quercetin (positive control) exhibited significant differences in their anti-angiogenic effects as shown in Figure 3. Similarly, significant differences were observed between groups treated with *A. squamosa* extract at doses of 500 ppm and 250 ppm, as well as 500 ppm and Quercetin (positive control). This suggests that *A. squamosa* leaf extract has significant anti-angiogenic potential, with the 250 ppm concentration being most effective.

DISCUSSION

In this study, the angiogenic potential of *A. squamosa* leaves was determined through CAM assay, one of the most widely used methods for detecting angiogenesis.[34] Quercetin, a natural flavonoid, was used as positive control since it has been shown to inhibit blood vessel formation and cancer cell growth.[35] Phytochemical analysis of *A. squamosa* leaves revealed the presence of flavonoids, alkaloids, phenolic compounds, and tannins, all of which are associated with anti-angiogenic and anticancer properties. Flavonoids, such as quercetin, are known to inhibit angiogenesis by targeting signaling pathways involved in vascular development.[36] Similarly, alkaloids isolated from *A. squamosa* have demonstrated potent cytotoxic effects against various cancer cell lines.[37] Phenolic compounds, including ellagic acid, have been shown to suppress angiogenesis by modulating oxidative stress and directly inhibiting VEGFR-2 signaling pathways.[38] The presence of multiple bioactive constituents in the extract likely contributes to its anti-angiogenic efficacy, while their interactions might also explain the non-linear dose-response observed.

The inhibitory effect on blood vessel formation of *A. squamosa* extracts in this study did not follow a dose-dependent pattern. The data reveal a non-monotonic, inverted U-shaped dose-response relationship, with maximal inhibition observed at 250 ppm, reduced efficacy at 500 ppm, and partial inhibition at 1000 ppm. Such non-linear dose-response patterns are characteristic of hormesis, wherein low doses elicit beneficial or stimulatory effects, while higher doses result in inhibitory or toxic outcomes.[39] These findings highlight the complexity of dose-response dynamics in plant-based therapies. The non-monotonic response could be attributed to several mechanisms. Differential receptor binding affinities and the activation of distinct signaling pathways at varying concentrations are plausible explanations. Additionally, synergistic or antagonistic interactions between the extract's constituents may influence the observed dose-dependent outcomes.[40] These complexities underscore the need for a comprehensive dose-response studies to better characterize the pharmacological profile of *A. squamosa* and

establish its optimal therapeutic range. Understanding the hormetic nature of the dose-response relationship observed in this study is critical for optimizing the therapeutic application of *A. squamosa*. Identifying the precise concentrations that provide maximal benefit with minimal adverse effects will inform dosing strategies and improve clinical outcomes.

The CAM assay, while a valuable model for evaluating angiogenesis, has limitations. The dense vascular network in the CAM can complicate the differentiation between true vascular inhibition and apparent reductions in vascular growth.[41] Thus, while these results are promising, further studies utilizing complementary models, such as tumor xenografts or advanced *in vitro* techniques, are necessary to validate the anti-angiogenic potential of *A. squamosa* and elucidate its mechanisms of action.

CONCLUSION

The present study aimed to perform phytochemical screening and evaluation of anti-angiogenic property of the acetone extract of *A. squamosa* leaves. The crude extract was found to possess significant anti-angiogenic properties, with the highest activity observed at a concentration of 250 ppm. These findings support the traditional usage of *A. squamosa* in ethnopharmacology as a potential remedy for various ailments, including those related to angiogenesis such as cancer. Nonetheless, it is imperative to conduct further investigations to identify specific compounds and explain the underlying mechanisms by which *A. squamosa* leaves inhibit vascularization and validate these findings through *in vivo* and clinical studies.

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Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

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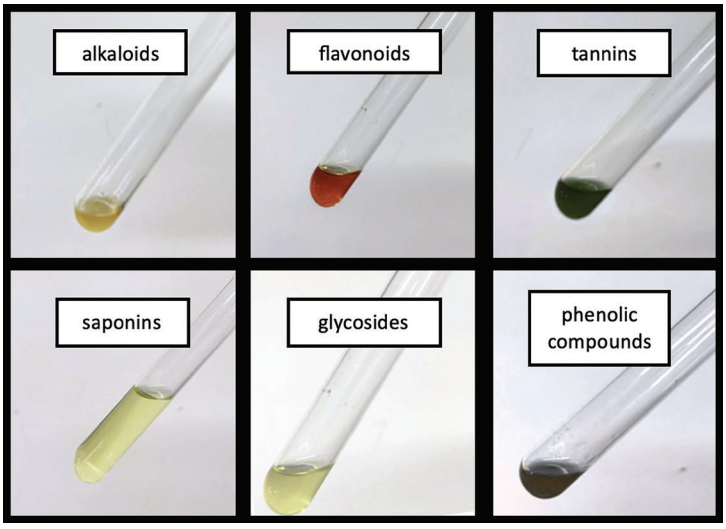
APPENDIX



Phytochemical test results of acetone extract of *A. squamosa* leaves.