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Assessing the Therapeutic Potential of Miracle Berry *(Synsepalum Dulcificum)* Leaf Extract in Attenuating Angiogenesis: Implications for Supportive Care in Cancer Treatment

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ABSTRACT

Angiogenesis is critical in cancer progression, and examining the anti-angiogenic effects of *Synsepalum dulcificum* leaf could influence cancer treatment. This study utilized distilled water (negative control), Vitamin A (positive control), and Miracle Berry Leaf Crude Extract at concentrations of 100mg/L, 1000mg/L, 5000mg/L, and 10000mg/L on 8-day-old duck eggs. Methods were through chick chorioallantoic membrane assay and morphometric analysis of embryos. The result shows that the treatment group showed smaller vascular densities compared to Vitamin A (169±31.0), with T3 being the lowest and exhibited a Cohen's d of 2.64. ANOVA also revealed significant differences ($p<0.05$) in vascular density. Meanwhile, T3 showed the highest vascular inhibition and effect size ($d=3.60$) and results showed non-significant p-values ($p>0.05$) across treatments. Furthermore, T1 showed the highest values in morphometric parameters while T3 and T4 had lower values demonstrated by the underdeveloped embryos. No significant differences were found across treatments and between the four concentrations and Vitamin A in terms of morphometric analysis. Results of low vascular densities indicate anti-angiogenic activity. It was evident that with increasing concentration, there was a decrease in blood vessel count and a greater inhibition which was likely due to phytochemicals present and the extract's anti-angiogenic effects. A non-significant value suggests that varying concentrations may have similar inhibitory effects. Morphometric analysis showed that higher concentrations of *S. dulcificum* led to underdeveloped embryos, with T3 being the most toxic. Statistical analysis indicated consistent anti-angiogenic effects, suggesting all concentrations impact anti-angiogenesis similarly, and that their effects are similar with Vitamin A.

Key words: Angiogenesis, cancer, Synsepalum dulcificum, supportive care.

INTRODUCTION

Background of the Study

The rising number of cancer cases and other non-communicable diseases (NCDs) poses major challenges for patients and healthcare systems globally. This trend threatens progress toward the 2030 Agenda for Sustainable Development, which aims to reduce deaths from the four main NCDs by one-third among people aged 30 to 70 by 2030 (Budreviciute et al., 2020; WHO, 2023). Beyond the shadow of doubt, accessible, affordable healthcare fosters better health, driving research towards natural, plant-based therapies for diverse diseases. Furthermore, the increasing threat of cancers resistant to medication highlights a critical necessity for developing more effective anticancer agents (Chandra et al., 2023). In the relentless pursuit of new cancer therapies, natural products have become vital for discovering novel biologically active compounds with distinctive structures and mechanisms. Their clinical significance is well acknowledged within oncology, especially as a viable alternative to traditional chemotherapy, which is frequently hampered by severe adverse effects and diminished efficacy resulting from multi-drug resistance (Dehelean et al., 2021). Angiogenesis becomes apparent in intricate researches in healthcare as an essential facet in the progression as well as management of disease, especially when dealing with cancer. According to Laschke et al. (2022), there is a substantial need for investigative methodologies that facilitate the exploration of angiogenic processes and the evaluation of therapeutic agents with pro- and anti-angiogenic properties.

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Cancer statistics are vital for understanding the disease's societal impact. These metrics, including incidence, mortality, and survival rates, quantify cancer's burden and inform public health strategies and clinical interventions (National Cancer Institute, 2024). According to the World Health Organization (2021), cancer caused nearly 10 million deaths globally, with the most common types being lung, breast, colon, and prostate cancers. In the 21st century, cancer is projected to become the leading cause of death, significantly affecting life expectancy. Over 60% of cases and 70% of deaths occur in Asia, Africa, and Central and South America (World Health Organization, 2019).

In the Philippines, lung cancer is the leading cause of cancer death, making cancer the third overall cause of mortality. According to a study by the University of the Philippines' Institute of Human Genetics, National Institutes of Health, cancer affects 189 out of every 100,000 Filipinos, with four Filipinos dying of cancer every hour, totaling 96 deaths daily (Flores, 2022). Additionally, recent data from the FHSIS Annual Report (2019-2022) of the Department of Health Center for Health Development – Caraga highlights concerning trends in cancer incidence and mortality in the Caraga region. From 2019 to 2022, 169 cancer cases were reported, with an increase from 21 cases in 2019 to 50 in 2020 and 2021, followed by a slight decrease to 48 in 2022. The data also reveals a gender disparity, with 36% of cases in males and 64% in females, aligning with national trends of higher cancer incidence in women. Tragically, the number of cancer-related deaths during this period reached 1,956, with significant fluctuations: 586 in 2019, 666 in 2020, 278 in 2021, and 426 in 2022. Here, a gender difference is evident, with 49% of deaths in males and 51% in females. With these said, the global burden of cancer is increasing, imposing significant physical, emotional, and financial challenges on individuals, families, communities, and healthcare systems. Many health systems in low- and middle-income countries are ill-equipped to handle this burden, resulting in many cancer patients lacking access to timely and high-quality diagnosis and treatment.

Flores (2022) claims that NCDs, like cancer, pose a "silent disaster" in the Philippines, causing 300,000 deaths annually, 800 per day, and 33 every hour. The human cost has been compared to "two 747 planes packed with passengers colliding every day." In addition to this fact, cancer's constantly changing nature, caused by uncontrollable cell growth brought about by mutations, contributes to the reason why it remains a key focus of research (Flores, 2022; National Cancer Institute, 2021). With cancer’s complex nature, treatment strategies are adjusted based on the cancer stage and advancement with the goal to either prevent further growth or improve the patient's quality of life (CDCBreastCancer, 2020). At present, there are therapies that could prevent the illness from getting worse. Cancer therapies have evolved significantly beyond conventional treatments like surgery, chemotherapy, and radiotherapy, and these recent advancements such as stem cell therapy, targeted therapy, and nanoparticles now play crucial roles in complementing these established methods (Debela et al., 2021)

Cancer is a genetic disease caused by changes to genes that regulate cell function, particularly growth and division. These genetic alterations lead to abnormal cell proliferation, resulting in over a hundred distinct types of cancer, each with unique behaviors and treatment responses (National Cancer Institute, 2021; Cooper, 2020). Since the body consists of trillions of cells, cancer can begin almost anywhere. Normally, cells grow and divide to form new cells, and old or damaged cells die. Sometimes, this process malfunctions, causing abnormal cells to grow and multiply uncontrollably. These cells can form tumors, which may be cancerous (malignant) or non-cancerous (benign). Malignant tumors invade nearby tissues and can spread to other parts of the body (metastasis), while benign tumors do not invade nearby tissues and usually do not return after removal, but they can sometimes grow large and cause serious problems, such as in the brain. (National Cancer Institute, 2021). Cancer progresses in different phases. According to Cooper (2020) and Aguilar-Cazares et al. (2019), it starts with initiation, which is when genetic changes emerge in a single cell or a small group of cells as a result of variables such as carcinogen exposure, genetic predisposition, or random errors during cell division. During the promotion stage, these altered cells begin to multiply abnormally, acquiring new genetic changes that promote their growth and survival. At this point, the tumor may still be benign. However, as the process progresses, the tumor becomes malignant as cells continue to grow uncontrollably and acquire more mutations, allowing them to infiltrate surrounding tissues and move to other areas of the body, a process known as metastasis. Advanced tumors are encircled by stromal and immune cells during carcinogenesis, which promotes tumor growth. Furthermore, from the start of carcinogenesis to the tumor in situ and later

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stages of the disease, inflammation and angiogenesis are processes that are crucial to the development of cancer. Vascular hyperpermeability during acute inflammation facilitates the infiltration of immune response cells, such as leukocytes and monocytes/macrophages, and inflammatory mediators at the site of damage. Vascular endothelial growth factor (VEGF) is a multifunctional molecule and growth factor that also plays a crucial role in controlling vascular permeability. According to National Cancer Institute (2018), angiogenesis is essential for cancer progression, as solid tumors require a blood supply to grow beyond a few millimeters in size. Tumors facilitate the formation of this blood supply by secreting chemical signals that promote angiogenesis. Furthermore, tumors can induce nearby normal cells to produce angiogenic signaling molecules. The newly formed blood vessels provide the growing tumors with oxygen and nutrients, enabling them to expand, invade adjacent tissues, disseminate throughout the body, and establish new colonies of cancer cells, known as metastases.

Angiogenesis, crucial for tissue homeostasis, requires proper regulation. Studying this process through animal models holds key to many biological conundrums, including therapeutic development (Prado et al., 2019). The chick embryo's chorioallantoic membrane (CAM) serves as a versatile, budget-friendly in vivo model for diverse fields like angiogenesis research and drug testing, xenografting, and cancer research, along with other scientific and commercial disciplines such as microbiology, biochemistry, cosmetics, and so on. This non-sentient model, which is used as an alternative to other mammal experimental models, aligns with the "3R" principles (Replacement, Reduction, and Refinement) promoting ethical and efficient animal research while addressing public concerns about animal welfare (Kundeková et al., 2021). Anti-angiogenic therapy is an old but promising strategy against cancer progression that tries to eliminate the nutrition and oxygen supply to tumor cells by decreasing the vascular network and avoiding the development of new blood vessels. However, its effectiveness can be limited by drug resistance, often caused by changes in the microenvironment of the tumor (Lopes-Coelho et al., 2021).

Medicinal plants with fewer side effects are paramount for humankind to cure various ailments compared to newly developed allopathic medicines (Jia et al., 2022). Research highlights the Miracle Berry *(Synsepalum dulcificum) fruit's* abundance in phytochemicals including alkaloids, saponins, flavonoids, polyphenols, cardiac glycosides, and anthraquinones, in addition to protein, fat, fiber, carbohydrates, vitamins, and other nutrients, as well as its potential to be a potent medicinal plant. This natural resource presents exciting possibilities for future medicinal applications. However, according to Angeles et al. (2021), the plant is distinguished by its slow growth and a gestation period of 2.5 years. This, therefore, makes the miracle berry fruit less accessible compared to the leaves of the plant which contains similar phytochemical compounds with the miracle berry fruit such as alkaloids, tannins, flavonoids, cardiac glycosides, saponins and anthraquinone, thus making it a good alternative (Awotedu & Ogunbamowo, 2019). Additionally, results from the study carried out by Ma et al. (2022), on the other hand, have also shown that 18 phenolic compounds were found in Miracle Berry Leaf (MBL) extract. Among the phenolics present in the extract, 3-O-p-coumaroylquinic acid, myricetin-3-O-rhamnoside, quercetin-3-D-galactoside, and quercetin-3-rhamnoside are the most abundant. And when tested at very low concentrations, the MBL extract effectively reduced the formation of subintestinal vein vessels in zebrafish while exhibiting minimal toxicity and high anti-angiogenesis action.

This study integrates the significance of healthcare accessibility, the challenges posed by NCDs, like Cancer, the role of angiogenesis in disease management, and the potential of Miracle Berry Leaf and its extract as promising alternatives in combating cancer, and incorporating cost-effective, accessible, and natural therapeutic options to enhance patient well-being, improve treatment adherence, and contribute to a holistic approach to healthcare offering a comprehensive framework for further exploration and development in the field of cancer treatment and prevention. The research outcomes may have implications for medical professionals and healthcare providers involved in managing angiogenesis-related disorders. Accumulating evidence indicates that a variety of diseases are dependent on angiogenesis, such as atherosclerosis, stroke, pulmonary hypertension, cardiac hypertrophy, diabetic retinopathy, aneurysms, lung cancer, and gastric cancer (Liu et al., 2020). Understanding the effects of Miracle Berry Leaf Crude extract on angiogenesis could potentially influence treatment approaches or serve as a complementary therapy in certain cases.

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The researchers selected this study due to its innovative yet novel use of Miracle Berry *(Synsepalum dulcificum)* Leaf Extract. By evaluating the effects of this extract on angiogenesis, uncovering potential therapeutic applications, specifically in reducing angiogenesis may be gained. Demonstrating its effectiveness in this area could pave the way for developing natural and economical supportive care treatments for cancer patients, aligning with the growing interest in accessible and cost-effective complementary therapies. The anticipated benefits of the study could have a significant impact on the broader community.

STATEMENT OF THE PROBLEM

The primary objective of this research study is to investigate the anti-angiogenic properties of Miracle Berry *(Synsepalum dulcificum)* Leaf Extract on the Duck Egg Chorioallantoic Membrane (CAM) Assay and Morphometric measurements of the Duck Egg Embryo. Specifically, the study will focus on addressing the following statements:

1. What secondary metabolites do the Miracle Berry *(Synsepalum dulcificum)* Leaf Crude Extract possess that have anti-angiogenic properties on Duck Egg Chorioallantoic Membrane (CAM) Assay?
2. What are the mean values of vascular density and vascular inhibition of the Chorioallantoic Membrane (CAM) Assay performed on duck egg embryos, with a focus on the four different concentrations of 100 mg/L, 1000 mg/L, 5,000 mg/L, and 10,000mg/L**;**
3. Are there significant differences in vascular density and inhibition in the CAM Assay at four different concentrations?
4. Is there a significant difference in vascular density between the experimental (Miracle Berry Leaf Crude Extract at four different concentrations) and control groups (Vitamin A)?
5. Is there a significant difference in vascular inhibition between the experimental (Miracle Berry Leaf Crude Extract at four different concentrations) and control groups (Vitamin A)?
6. What is the mean growth of the duck egg embryo using the Miracle Berry *(Synsepalum dulcificum*) leaf extract at varying concentrations using morphometric analysis in terms of:

a. Embryo Weight (EW)
b. Crown-Rump Length (CRL)
c. Head-Beak Length (HBL)
d. Forelimb Length (FL)
e. Hindlimb Length (HL)
f. Eye Diameter (ED)

7. Is there a significant difference in the mean growth of the duck egg embryo at four concentrations?
8. Is there a significant difference in the growth of the duck egg embryo among the four concentrations of Miracle Berry Leaf Crude Extract and Vitamin A.

Hypothesis

Ho1. There is no significant difference on the vascular inhibition and density on the Chorioallantoic Membrane (CAM) Assay in terms of the four concentrations.

Ho2. There is no significant difference in the vascular inhibition between the experimental and control group (Vitamin A).

Ho3. There is no significant difference in the vascular density between the experimental and control group (Vitamin A).

Ho4. There is no significant difference between the mean growth of the duck egg embryo in terms of the four concentrations.

Ho5. There is no significant difference in the growth of the duck egg embryo between the four concentrations of Miracle Berry Leaf Crude Extract and Vitamin A.

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SIGNIFICANCE OF THE STUDY

The study aims to investigate the anti-angiogenic properties of Miracle Berry (*Synsepalum dulcificum*) Leaf extract using the duck egg chorioallantoic membrane (CAM) assay. By examining the effects of miracle berry leaf crude extract on angiogenesis, the study seeks to determine if the extract has the potential to inhibit or reduce blood vessel formation, which is one of the major mechanisms of cancer growth. The findings of this research endeavor can expand our knowledge of the therapeutic potential of Miracle Berry *(Synsepalum dulcificum)* Leaf and facilitate advancements in the field of angiogenesis research and treatment modalities. Benefitting the study will be the following sectors:

Cancer patients. Understanding the anti-angiogenic potential of Miracle Berry Leaf Crude extract has specific relevance for cancer patients with tumors which heavily rely on angiogenesis for their growth and spread. This extract could potentially be explored as a complementary therapy alongside conventional cancer treatments to increase the effectiveness of the existing treatment and potentially minimize certain side effects.

Department of Health (DOH). In line with the Department of Health's 10 approved Herbal Medicines, the research findings of this study contributes to new knowledge on a potential addition to the medicinal plants endorsed by the DOH therefore, expanding the public's access to safe and effective herbal medicines and constructing healthcare policies based on evidence-based research which will further lead to a healthcare system built on effectiveness and efficiency.

Future Researchers. The study provides insights into the anti-angiogenic properties of miracle berry extract, contributing to further research on its potential medicinal applications, plant-based therapeutics, and morphometric analysis techniques. The findings serve as a basis for future research, inspire new studies, and stimulate scientific discussions and collaborations. Furthermore, modifying nursing research and education to meet the changing needs of the Filipino people.

Department of Science and Technology (DOST). The study provides opportunity for profound inquiry and understanding in the field of angiogenesis and may even boost interest in initiating researches in the use of local medicinal plants in healthcare practices. In line with this, advancements in the current known facts on the subject of possible uses of medicinal plants such as the Miracle Berry leaf in angiogensis and its other uses will be cultivated and may motivate more researches to be realized. With opportunities of research fundings and interest from the DOST, novel approaches and findings from research endeavors on angiogenesis and therapeutic properties of local medicinal plants like the Miracle Berry will contribute significant benefits to healthcare and bring forward the frontiers of medical research and public health.

Healthcare Professionals. Research such as this may aid in further cultivating a culture of promoting life-long learning among healthcare professionals. This may also grow as a possible field of interest that would encourage an individual in the profession to pursue a research study on other possible uses of medicinal plants available in the country. Furthermore, this research would provide insights among healthcare professionals with regards to possible cost-effective approaches and opportunities for their patients thereby, being pro-active patient advocates for individuals who may be suffering from health conditions requiring high expenditures.

Academic Institution. This study may encourage Father Saturnino Urios University to create its own Institutional Animal Care and Use Committee (IACUC) to cater more studies of the like, thus shedding more light and opportunities for students to engage and pursue experimental research studies involving live animals. Establishing an IACUC at the university would highlight its commitment to advancing scientific knowledge while ensuring animal welfare. This move would attract passionate students and faculty, positioning the institution as a leader in responsible research. Ultimately, it could lead to significant discoveries and innovations, benefiting the academic community and society.

Local Government Unit’s Sangguniang Panlungsod - Committee on Science and Technology. As angiogenesis research continues to grow as a field of interest in research studies, the research findings of this study may play a significant role in contributing and supporting the local government's efforts and dedication to fostering technological and research advancements in the city. The findings drawn from the application of this research

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might encourage greater interest among the city's local leaders to support and invest more in the cutting-edge scientific endeavors of research. With this, researchers would have the means to explore new ideas and develop innovative solutions, creating a vibrant scientific community. This investment in research not only benefits the scientists and academics but also helps to establish our city as a hub of medical and technological innovation.

Local Government Unit's Sangguniang Panlungsod - Committee on Health and Sanitation. The findings from this research could potentially bring greater interest from the local government in exploring medicinal plants, such as the Miracle Berry and those with possible health benefits and are readily available in the city. These may hold key compounds that could contribute to effective cancer treatments. By focusing on locally sourced, natural remedies, the government could support both scientific advancement and the utilization of regional resources. This approach not only fosters a sense of community but also positions the city as a leader in innovative and sustainable healthcare solutions. Encouraging investment may lead to significant breakthroughs, ultimately benefiting the health and well-being of Butuanons and setting a precedent for other regions to follow.

SCOPE AND LIMITATIONS OF THE STUDY

The study was conducted within a specific timeframe. The pre-experimental phase began in March 2024, during which the collection, washing, and drying of leaves, along with phytochemical analysis, were carried out. On June 6, 2024, the crude leaf extract was produced and diluted at Caraga State University - Main Campus Biology Department Laboratory. The experiment proper took place on June 7 and 10, 2024, at the Department of Agriculture - Trento, involving the application of treatment, harvesting of duck embryos, morphometric analysis, and blood vessel counts.

The primary focus of this study was to determine the anti-angiogenic properties and activity of Miracle Berry (*Synsepalum dulcificum*) leaf crude extract using the Duck Egg Chorioallantoic Membrane (CAM) Assay. A comprehensive phytochemical analysis was conducted on the leaf extract to identify and quantify its components, using the Base-Smith and Metcalf Method for flavonoids, the Froth test for saponins, the Liebermann-Burchard Test for steroids, Dragendroff's test for alkaloids, and the Ferric Chloride Test for tannins. These qualitative phytochemical tests presented a methodological limitation, as results may vary with other tests. Additionally, the isolation of leaf components was not performed due to the lack of facilities and expertise available in Butuan City.

This study was only utilized on eighth-day (*Anas platyrhynchos*) duck eggs purchased from Jinayon Duck Farm, Trento, Agusan del Sur, and not on a large-scale farm. Four distinct concentrations of Miracle Berry leaf extract (100 mg/L, 1000 mg/L, 5,000 mg/L, and 10,000 mg/L) were used in the CAM Assay to evaluate the extract's effects on vascular density and inhibition; significant decrease in these parameters indicated anti-angiogenic activity. Simultaneously, the growth parameters of duck egg embryos, including duck egg weight (DEW), embryo weight (EW), Crown-Rump Length (CRL), Head-Beak Length (HBL), Forelimb Length (FL), Hindlimb Length (HL), and Eye Diameter (ED), were assessed using morphometric analysis. Statistical analysis was used to identify notable variations in vascular inhibition and density among the concentrations compared to a control group. Ethical considerations and limitations were addressed to ensure the research aligned with ethical standards.

This study did not directly administer Miracle Berry leaf crude extract to cancer cells, focusing instead on the effects on eighth-day duck eggs. Therefore, further research on the direct effects on cancer cells, including potential therapeutic benefits or cytotoxic effects, is needed. Additionally, human samples were not used for experimental treatment, as this research was still in its first phase. The applicability of these findings to human health or their potential for translation into therapeutic interventions is limited at this stage. Further research, involving direct tests on human cells and tissues, will be essential to advance our knowledge and identify potential therapeutic uses in the future.

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METHODOLOGY

Design

This study employs a quantitative prospective experimental research design on the evaluation of the anti-angiogenic properties of miracle berry *(Synsepalum dulcificum)* leaf crude extract using duck egg chorioallantoic membrane (CAM) assay. This design is termed as prospective as it is focused on gathering information, forward in time, on subjects after their exposure to treatment (Ranganathan & Aggarwal, 2018). Specifically, it follows a parallel group design which involves assigning each subject to a group, and an intervention treatment will be applied to the experimental group while the other will receive a controlled treatment. This allows the researchers to draw conclusions about the effectiveness of the treatments by observing the differences between the groups with different treatments in the same time frame and setting (Turner, 2020). This makes it possible to compare the anti-angiogenesis treatment's effects on angiogenesis and CAM formation directly. To reduce bias and guarantee that any detected variations between groups are most likely the result of the therapy rather than pre-existing characteristics, subjects are selected based on an inclusion criterion explained in Figure 10, and are then grouped by their weight. With that, this design is the most appropriate for the study as it compares two or more treatments and explores on the cause-and-effect relationship between the variables in Figure 1. The future result of the study will determine the effects of anti-angiogenic agents coming from an organic sample.

MATERIALS AND METHODS

Flowchart Diagram

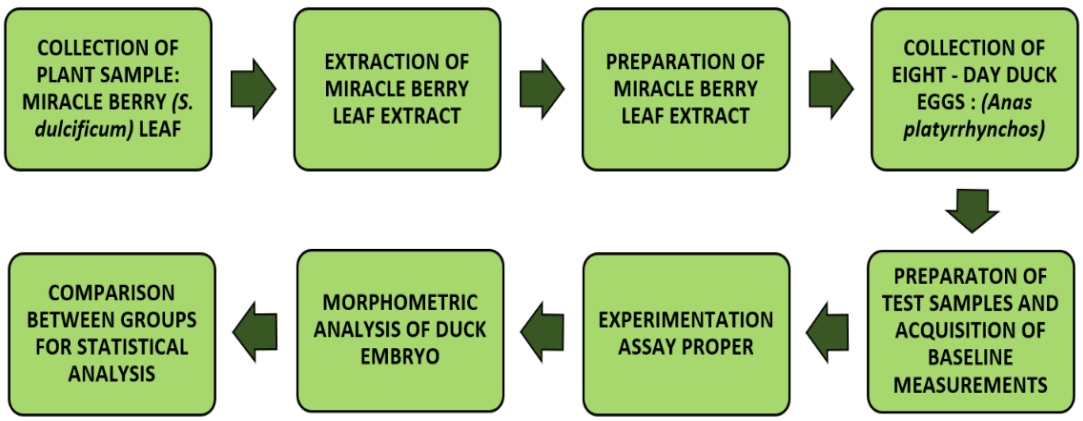

Figure 2. Flowchart diagram of the methodology

Preparation

Collection of Plant Sample and Extraction

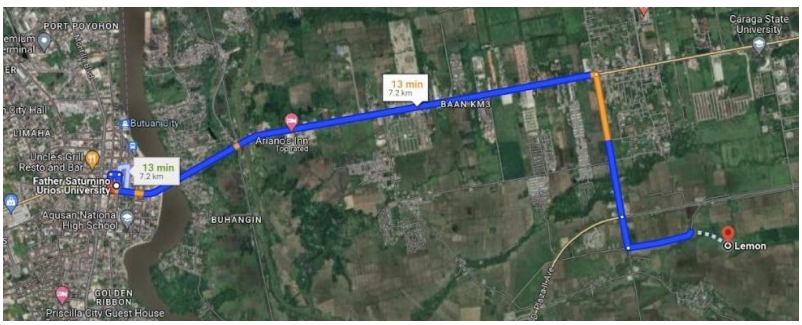

Figure 3. Barangay Lemon, Butuan City Location

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Two kilograms of Miracle Berry (*Synsepalum dulcificum*) leaves were locally sourced from Barangay Lemon, Butuan City. Once collected, the researchers followed the the methodology for extraction of medicinal plants by Bandiola (2018). The leaves were immediately washed with running tap water, and then rinsed with distilled water as it is recommended to use when rinsing plant tissues to prevent the introduction of mineral or microbial contaminants that could interfere with experiments (Kozlowski & Pallardy, 2020). The leaves were then subjected to air-drying in one of the researchers' houses, inside a room, under a shade with no contact to direct sunlight at room temperature for two weeks, Within that window of two weeks, the leaves were monitored daily and were remained untouched. This method of drying prevented the leaves from withering from sun exposure and losing their light-sensitive bio actives (Bandiola, 2018).

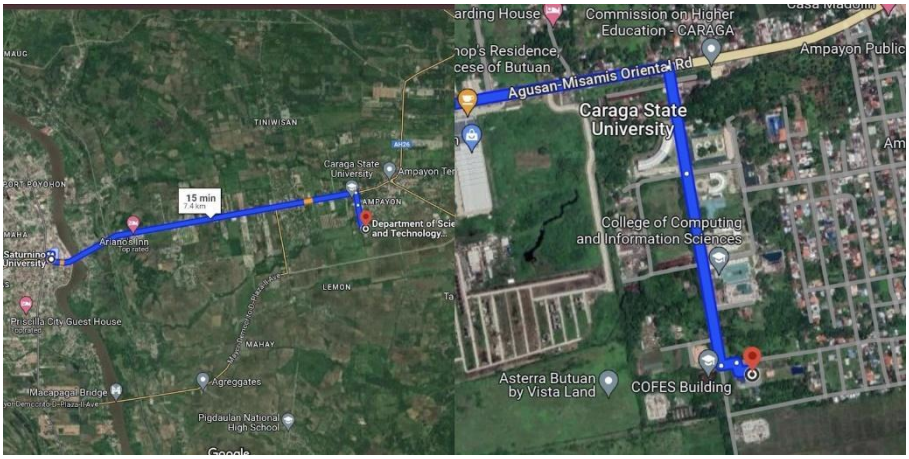

Figure 4. Department of Science and Technology Caraga location

The researchers then sent the leaves to the Department of Science and Technology Caraga Region, located at the Caraga State University - Main Campus, Ampayon, Butuan City, to turn the leaves into an ethanolic leaf extract. The dried leaves were pulverized with an electric blender. The total weight of the pulverized leaves of 250g was then transferred to a sterilized glass container containing 1L of 80% ethyl alcohol for 72 hours at room temperature. This was then filtered using an ordinary filter paper and concentrated at 60°C using a rotary evaporator which was placed in a steam bath until the ethanolic leaf extract was produced.

Qualitative Analysis for Phytochemical Components

The ethanolic leaf extract then underwent phytochemical screening. A residue from the miracle berry ethanolic leaf extract after its production was reconstituted with 10 ml of 50% ethyl alcohol for the presence of secondary metabolites using the standard methods from the book of Claustra et al. (2006) titled, A guidebook to plant screening: Phytochemical and Biological.

Test for Flavonoids (Bate-Smith and Metcalf Method)

0.5 concentrated hydrochloric acid was mixed with the extract and then placed in a warm water bath for five minutes. If the solution changed into a red violet color, this indicated a positive result.

Test for Saponins (Froth test)

10 milliliters of distilled water was diluted to the extract. It was then shaken for five minutes. If there was a formation of a stable foam, this indicated the presence of saponins.

Test for Steroids (Liebermann-Burchard Test)

0.3 milliliters of acetic anhydride was added to the extract and mixed gently followed by a drop of concentrated sulfuric acid. If the solution changed to a green or blue-green color after a few minutes, this indicated a positive result.

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Test for Alkaloids

A preliminary Test was done first. 1 milliliter of Dragendorff's reagent was added to the test tube. If an orange-red precipitate was formed, this indicated the presence of alkaloids.

A confirmatory Test was then done. A dropwise of 28% ammonia was added into the test tube until the solution was alkaline to the litmus. The alkaline solution was then extracted three times with the addition of ten milliliters of chloroform. The lower chloroform extracts were then combined and the upper aqueous layer was reserved (for the test for quaternary and/or amine oxide basis). The chloroform extracts were then evaporated under the hood and over a steam bath until it dried. The residue was then mixed with 5 milliliters of 2M hydrochloric acid, stirred over a steam bath for two minutes, and then cooled down. The mixture is then filtered, divided into two equal portions, and then tested the same way in the preliminary test. If the solution's relative amount of precipitation had a slight turbidity (+), definite turbidity (++), or heavy precipitation (+++), it indicated the presence of primary alkaloid, secondary alkaloid, and tertiary alkaloid, respectively.

Test for Quaternary and amine oxide bases

The upper aqueous layer reserved during the confirmatory test for the alkaloid, as stated above, was then acidified with 2M hydrochloric acid. The solution was then filtered, divided into two parts, and then tested the same way as the preliminary test for alkaloids. If the solution's relative amount of precipitation had a definite turbidity (++) or heavy precipitation (+++), this indicated a positive result. However, if it had a slight turbidity (+), this indicated a negative result,

Test for Tannins (Ferric Chloride Test)

A few drops of 10% ferric chloride solution was mixed with the extract. If the solution turned into a brownish-green color, this indicated the presence of condensed tannins. If it turned into a blue-black color, this indicated the presence of hydrolysable tannins.

Test for glycosides (Keller-Kilani test)

A mixture of one drop of 2% Iron Chloride ($FeCl_3$) and 4 milliliters of glacial acetic acid was added to the extract, followed by the addition of 1 milliliter of concentrated Sulfuric Acid (H_2SO_4). If there is a formation of brown rings in between the layers, it indicated a positive result.

Preparation of Miracle Berry Leaf Extract

Figure 5. Caraga State University-Main Campus Biology Department Laboratory Location

The researchers then utilized the instruments in Caraga State University- Main Campus Biology Department Laboratory in the production of the crude leaf extract. A small beaker contained with 70mL of the Miracle Berry Leaf Extract was heated on a hot plate for 4 hours at 80°C, then oven-dried for 16 hours overnight at 30°C . The day after, the extract was heated again on a hot plate for 1 hour and 30 mins at 80°C. This is to ensure that only ethanol is evaporated in the ethanolic leaf extract. The extract was stirred with a glass rod until it reached a viscous consistency, indicating that the ethanol has completely evaporated and leaving behind a

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pure extract of the Miracle Berry Leaf. Then, the treatments for each group were prepared, with each treatment in the experimental group being placed in a small tube and afterwards weighed on an analytical balance scale. The required volumes of distilled water were dispensed into small tubes of each treatment using a micropipette to achieve the desired concentrations for each treatment.

Specifically, the extract was diluted to 100 mg/L (T1), 1000 mg/L (T2), 5,000 mg/L (T3) and 10,000mg/L (T4) in accordance with the study of Balogun, F. O., & Sabiu, S. (2021) as experimental groups, while the negative control group with distilled water, and the positive control group with Vitamin A 25, 000 International Unit (IU). The Vitamin A used is Food and Drug Administration (FDA) approved. To dilute the extract for the experimental groups, the researchers calculated for the initial concentration by diluting 5.432 g which is the mass of the crude leaf extract after the ethanol was evaporated and weighed on an analytical balance scale, to a 100mL distilled water. This concentration was then calculated in mg/L (milligrams per liter), which is equivalent to parts per million (ppm) for water solutions by multiplying it to 1, 000, 000. After getting a value of 54, 320 mg/L, it is then divided the desired concentration with the initial concentration and then multiplied to the desired final volume of 5mL to get the volume of extract. Lastly, the volume of extract was subtracted from 5mL to get the volume of solvent (distilled water) needed.

$$\text{Initial Concentration} = \frac{\text{Mass of extract (g)}}{\text{Volume of distilled water (mL)}} \times 1{,}000{,}000$$

$$= \frac{5.432\text{g}}{100mL} \times 1{,}000{,}000$$

$$= 54,\ 320 \text{ mg/L}$$

$$\text{Solution Dilution} = \frac{\text{Desired Concentration}}{\text{Initial Concentration}} \times \text{Desired Final Volume}$$

$$= \frac{100 \text{ mg/L}}{54{,}320\ mg/L} \times 5\text{mL}$$

$$= \mathbf{0.0092\ mL} \text{ volume of extract}$$

$$\text{Volume of Distilled Water} = 5\text{mL} - 0.00920$$

$$= \mathbf{4.9908\ mL}$$

$$\text{Solution Dilution} = \frac{\text{Desired Concentration}}{\text{Initial Concentration}} \times \text{Desired Final Volume}$$

$$= \frac{1000 \text{ mg/L}}{54{,}320\ mg/L} \times 5\text{mL}$$

$$= \mathbf{0.0920\ mL} \text{ volume of extract}$$

$$\text{Volume of Distilled Water} = 5\text{mL} - 0.0920$$

$$= \mathbf{4.908\ mL}$$

$$\text{Solution Dilution} = \frac{\text{Desired Concentration}}{\text{Initial Concentration}} \times \text{Desired Final Volume}$$

$$= \frac{5{,}000 \text{ mg/L}}{54{,}320\ mg/L} \times 5\text{mL}$$

$$= \mathbf{0.4602\ mL} \text{ volume of extract}$$

$$\text{Volume of Distilled Water} = 5\text{mL} - 0.4602$$

$$= \mathbf{4.5398\ mL}$$

$$\text{Solution Dilution} = \frac{\text{Desired Concentration}}{\text{Initial Concentration}} \times \text{Desired Final Volume}$$

$$= \frac{10{,}000 \text{ mg/L}}{54{,}320\ mg/L} \times 5\text{mL}$$

$$= \mathbf{0.9204\ mL} \text{ volume of extract}$$

$$\text{Volume of Distilled Water} = 5\text{mL} - 0.9204$$

$$= \mathbf{4.0796\ mL}$$

Figure 6. Calculations of diluting the different experimental groups.

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Collection and Preparation of Duck Eggs

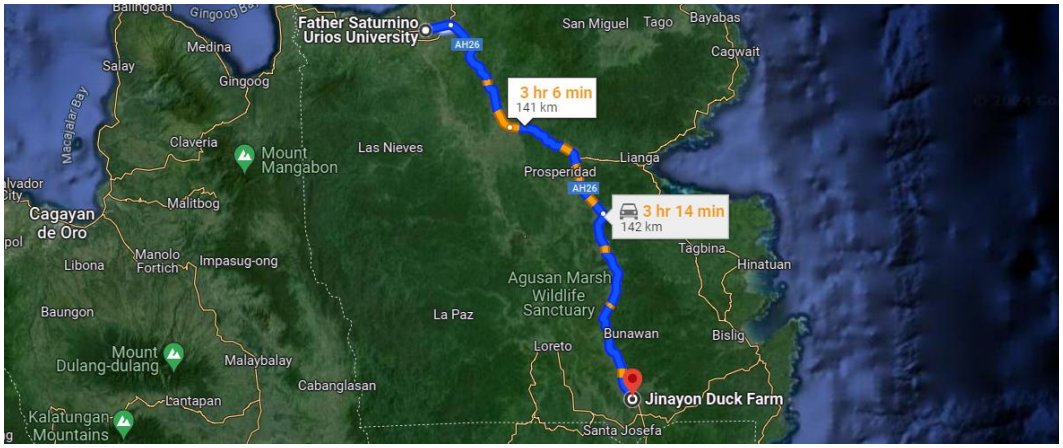

Figure 7. Barangay Jinayon Duck Farm, Trento, Agusan del Sur, location

Eight-day duck eggs and embryos were collected as a medium for this experiment which was obtained from Jinayon Duck Farm, Trento, Agusan del Sur. Eight-day Duck eggs and embryos were used since the developing Chorioallantoic Membrane (CAM) of this age, which is where angiogenesis takes place over the course of incubation, are well-developed and have reached the peak of CAM neovascularization (Aventurado et al., 2020). The CAM lacks full immunocompetence (Marshall et al., 2020); immune cells are still in the early stages of development, with full immunocompetence only established at around Day 18. Thus, using eight-day embryos allows us to observe CAM reactions with minimal influence from adaptive immune responses. The duck eggs were sanitized using 70% ethanol to remove dirt and other debris that could infect the eggs and cause mortality once embryos were opened. Three fertile eight-day duck eggs were utilized for both of the sets namely: controlled group and the experimental group, 100 mg/L (Treatment 1), 1000 mg/L (Treatment 2), 5,000 mg/L (Treatment 3), and 10,000mg/L (Treatment 4). The process of identifying the fertile duck eggs is found in the design technique found in page 97.

Actual Experimentation

Figure 8. Department of Agriculture - Trento Research and Experiment Station location

The researchers acquired an Animal Research Permit from the Bureau of Animal Industry (BAI) as shown on Appendix B, which allows the researchers to perform the experiment using the eight-day old duck eggs. In the process of acquiring it, the researchers held the experiment in the Department of Agriculture - Trento Research and Experiment Station, located at Barangay Poblacion, Trento, Agusan del Sur, under the supervision of a veterinarian to ensure that the rules and regulations on the Scientific Procedures Using Animals are adhered and complied.

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Assay Proper

The effects of *Synsepalum dulcificum* isolated extract on the angiogenesis activity in the chorioallantoic membrane (CAM) were evaluated. The concentrated extracts were dilluted to 100 mg/L (T1), 1000 mg/L (T2), 5,000 mg/L (T3) and 10,000mg/L (T4) concentrations of miracle berry *(Synsepalum dulcificum)* leaf crude extract as the experimental group. While the positive control group with Vitamin A, and the Negative control group with distilled water.

A 5 mm × 5 mm window was be opened just above the embryo on the egg's surface. A volume of 100 U/L was pipetted onto the surface of each duck egg's chorioallantoic membrane (CAM) in each treatment. The eggs were then wrapped in parafilm and incubated for 72 hours at 37°C and 65.5% humidity. To achieve a validated result for the study, a triplicate was performed.

Between days 8 and 10, the growing CAM vasculature begins to respond to additional proangiogenic stimuli. Duck eggs at this time are receptive to anti-angiogenic factors, which is why eight-day-old duck eggs (*A. platyrhynchos*) were chosen as the topic of the experiment. After 72 hours, the CAMs were collected by re-opening the sealed parts, exposing the CAM widely, and extracting the soft membrane covering the embryo intact, and each CAM was photographed three times.

Branched points were then collected and documented using the IKOSA software, which is a specialized software that can analyze blood vessels on CAM images, which makes it easy to gather and preserve landmark data from digital pictures (Rohlf, 2023). The CAM Assay Application is an AI-powered software tool for automatically quantifying angiogenic activities in CAM pictures. Following selection, pictures from each treatment group were submitted. The software analyzed each image for data characteristics to see if the requirements for running the CAM Assay Application, such as image file format, size, resolution, and color scheme, were met. After the images were uploaded, the program ran them through a visualization process in which portions of the discovered blood vessels were depicted in blue, vessel pathways were displayed as green lines, and vessel branching points were marked by red dots. Once each image had been analyzed, the data was exported in a suitable format for further study, such as CSV, and the processed images with branch points overlayed were downloaded. Vascular inhibition was then determined using the provided formula below (Vergara et al., 2021). (Refer to the figure below).

$$vascular\ inhibition = \frac{(branch\ points\ of\ negative\ control) - (branch\ points\ of\ the\ treatment)}{branch\ points\ of\ negative\ control}\ x\ 100$$

Figure 9. Formula of vascular inhibition

Morphometric Analysis

The embryos were then isolated from the egg and were measured using a digital balance to collect data on their weights. The morphometry of each embryo designated for the experiment was assessed utilizing a vernier caliper with centimeter (cm) as the measurement unit. The measurements were then documented by the researchers in a table as shown in Appendix C. The following metrics were assessed: The following measurements were taken: (1) Embryo Weight (EW); (2) Crown-Rump-Length (CRL), the distance from the top of the skull to the midpoint between the buttocks; (3) Head Beak Length (HBL), the distance from the back of the head to the tip of the beak; (4) Forelimb Length (FL), the distance from the connection point of the forelimb to the tip of the forelimb; (5) Hindlimb Length (HLL), the distance from the connection point of the hindlimb to the tip of the recognizable hindlimb; (6) Eye Diameter (ED), the edge-to-edge measurement of the duck embryos' eyeball.

Design Technique

In this study, eight-day *Anas platyrhynchos* (Kundeková et. al, 2021), duck eggs were used. The duck eggs were obtained from one reliable local supplier in Jinayon Duck Farm, Trento, Agusan Del Sur, in order to limit the potential for sampling bias.

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In order to minimize the influence of extraneous factors that could affect the results, the researchers utilized purposive sampling and established specific inclusion and exclusion criteria for duck egg embryos. This will enhance the internal validity and reliability of the study. Duck eggs must be 8 days of age to be included, so that developmental suitability can be verified by analysis. In order to ensure viable growth and an appropriate response to treatment, only fertile embryos that have been confirmed by candling performed by the researchers shall be considered valid. The duck eggs were weighed before any manipulations or the application of any treatments in order to establish a baseline and uniform weight for all the duck eggs before subjecting them to any experimental conditions. Furthermore, the embryos were screened to ensure they had no visible defects or abnormalities that could compromise development or interfere with test results.

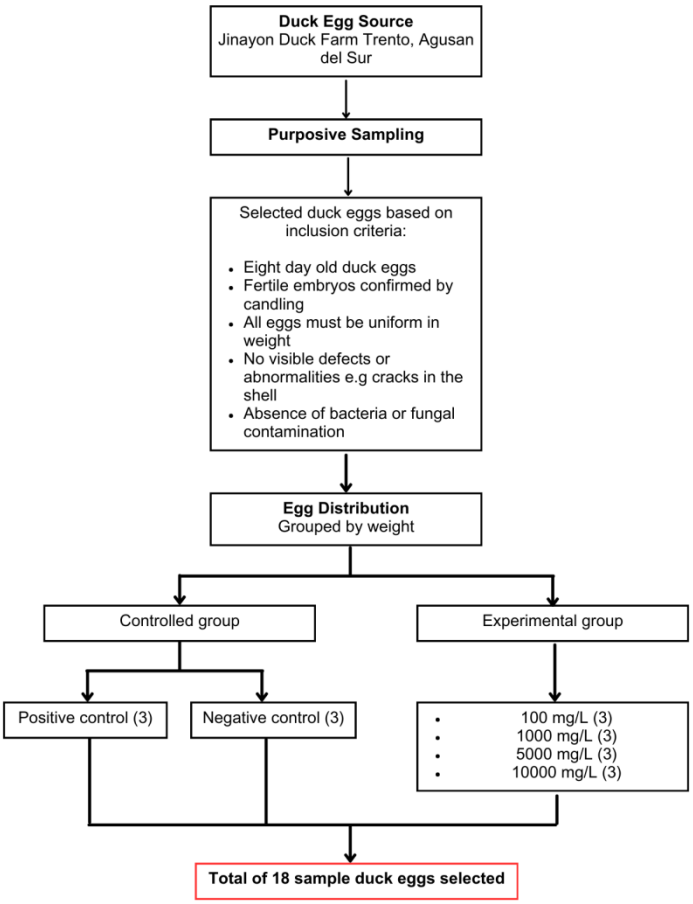

Figure 10. Schematic diagram of the design technique used to select duck eggs

On the contrary, to eliminate duck eggs that did not meet certain standards, the researchers established exclusion criteria. The study excluded undeveloped or dead embryos that had been identified during the candling procedure. Eggs that had incurred shell damage or showed other signs of damage potentially compromising embryo integrity were also excluded. Additionally, visual inspection was done to examine any abnormal appearances, such as discoloration, mold growth, or unusual odors, which may indicate bacterial or fungal contamination and were excluded to prevent potential interference with the experiments. Finally, eggs not precisely eight days old were excluded to maintain consistent developmental phases and treatment responses. These criteria collectively contribute to the rigorousness and reliability of experimental design.

The duck eggs were randomly assigned to the experimental and control groups, meeting the inclusion criteria. Initially, all duck eggs were carefully weighed individually. After weighing, the eggs were grouped according to their weights, with eggs of similar weight placed together in each group. Each weight group of eggs was then assigned to one of the concentration groups accordingly. This systematic approach ensured that each group of eggs was comparable in weight, maintaining consistency and reducing potential biases in the experimental setup. The duck eggs were then sanitized using 70% ethanol to remove dirt and other debris that could potentially infect the embryos and lead to mortality.

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Ethical Considerations

In the conduct of the research study, several key ethical considerations were emphasized and implemented to minimize harm and maximize scientific benefit, ensure the protection of scientific integrity, and build trust with the public. The researchers additionally took into account the notion that the utilization of animal samples for research purposes is both complicated and multifaceted, frequently requiring striking a careful balance between the well-being of the animals concerned and the possible advantages of the study. With that, prior to conducting the experiment, the researchers obtained the appropriate certifications and clearances from the Bureau of Animal Industry (BAI) and was exempted to obtain clearances from the Institutional Animal Care and Use Committee (IACUC) as the researchers will only be utilizing eight-day old duck eggs.

Additionally, the 3R Framework is the main moral guideline to take into account as the 3R Rule involving Replacement, Reduction, and Refinement is standardized and implemented for the scientific and research community to adhere to for the conduct of human-animal research studies. It has also helped emphasize public awareness of the welfare of animals along with the ethical considerations related to using animal samples in experiments. Lastly, to maintain the scientific integrity of the study, in gathering, analyzing, and reporting data, the researchers of this study aimed for accuracy and veracity, therefore preventing prejudice and data manipulation as a result.

Statistical Analysis

In this study, several statistical tools were employed to analyze the anti-angiogenic potential of *Synsepalum dulcificum* crude leaf extract. One-Way ANOVA (Welch's) was used to ascertain the presence of significant variations in the measurements among the different treatment groups. The statistical software Jamovi 2.3.24.0, licensed under AGPL3, was utilized for the satistical analysis. A p-value of less than 0.05 was considered to indicate the presence of significant differences. The test was selected because of its ability to withstand variations in group variances. Assumptions of ANOVA include normality, homogeneity, and independence. Before performing the ANOVA, the Shapiro-Wilk test (Table 2) was used to assess the normality of the data distributions. For vascular inhibition and density, the Shapiro Wilk test was done to test for normality and the data tested normal. This satisfies the normality assumption. However, in the morphometric analysis, the Shapiro-Wilk test for normality cannot be performed because of the lack of data due to the incomplete measurements from the underdeveloped embryos. Consequently, the researchers employed the Kruskal-Wallis Test, a non-parametric technique which was employed to evaluate differences among the groups; a p-value less than 0.05 showed significant differences among the groups. Furthermore, Cohen's D was calculated to quantify the effect size of the treatments relative to a control group; values of 0.2, 0.5, and 0.8 were interpreted as small, medium, and large effects, respectively. In order to determine specific pairwise differences between treatment groups, Tukey's Range Test was used as a post-hoc analysis after the ANOVA. Pairwise comparisons with p-values less than 0.05 revealed significant differences between those particular groups.

Table 2. Normality Test (Shapiro-Wilk)

	W	**p**
Embryo Weight	0.944	0.332
Blood Vessel Branching Count	0.926	0.166
Vascular Density	0.926	0.166

Note: A low p-value suggests a violation of the assumption of normality.

The data utilized in the statistical analyses for vascular density and vascular inhibition were obtained using the IKOSA Prisma software. IKOSA made it easier to analyze the CAM test in detail and concentrated on the important factors that were necessary to assess angiogenesis. These included vessel branching points, which indicated the locations of blood vessel splits, vessel area, which measured the total area covered by vessels in

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square pixels, mean vessel thickness, which computed the average vessel thickness in pixels, and total vessel length, which summed the length of all detected vessels in pixels. These numbers all have to be bigger than or equal to zero. To guarantee reliable results, a number of image quality conditions had to be satisfied prior to performing the analysis with IKOSA Prisma. The software can support a number of picture formats, including z-stack, time series, multichannel, and 2D (standard and/or entire slide images). With a color depth of eight bits per channel, images might have one gray channel or three RGB color channels. While whole slide images (WSI) might have different sizes, standard images should not have been more than 25,000 by 25,000 pixels. Typically, the resolution was between 1 and 7 micrometers per pixel. Vessels needed to be highly focused in the photos and their thickness needed to be between 3 and 220 pixels for the best detection results. The graphic output produced by the software consisted of red dots denoting vessel branching sites, green lines showing vessel pathways, and blue overlays depicting vessel regions.

Statistical Treatment

In this study the statistical tool that is utilized in the analysis, interpretation of data, and to test the null hypothesis of the proposed study involve One-Factor ANOVA, Kruskal-wallis test, Tukey's Range Test, and Cohen's D.

The software IKOSA is utilized to aid in counting the CAM branch points automatically. Statistical Tools/ Test are as follows:

- One-Way ANOVA (Welch's)
- Kruskal-Wallis Test
- Tukey's Range Test
- Cohen's D

RESULTS AND DISCUSSION

In this chapter, the researchers will present and analyze the study's findings in relation to its primary objective. To identify the phytochemical constituents of Miracle berry (*Synsepalum dulcificum*) leaves, phytochemical screening was performed. Given the following tests the miracle berry was seen to have obtained the presence of quaternary bases and amine oxides, steroids, and saponins as shown in Table 3.

Table 3. Phytochemical Screening results of Miracle Berry Leaf Extract

Secondary Metabolites

Parameter	**Presence or Absence**
Alkaloid Confirmatory Test (+) primary alkaloid (++) secondary alkaloid (+++) tertiary alkaloid	 -
Test for Quaternary Bases & Amine Oxide	+
Steroids Keller-Killiani Test: 2-deoxysugars	+

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Liebermann-Burchard Test: For Unsaturated Steroids	+
Flavanoids Bate-Smith & Metcalf Method: For Leucoanthocyanins	-
Saponins Froth Test	+
Tannins Ferric Chloride Test *Brownish-green color indicates the presence of condensed tannins *Blue-black color indicates the presence of hydrolysable tannins	-

The successful extraction of bioactive compounds from plants depends heavily on the chosen solvent. An ideal solvent should evaporate easily, be safe, and preserve the compounds without altering them. Factors influencing solvent selection include the type of compounds to be extracted, the desired extraction speed, and safety considerations for both the extraction process and the final product (Velavan, 2015). This might explain the presence of only quaternary bases, amine oxides, steroids, and saponins in our phytochemical study compared to other investigations, such as Awotedu (2021). The latter study, using ethyl acetate, ethanol, and methanol extracts, identified a high flavonoid content in *S. dulcificum* leaves along with phenolic compounds, alkaloids, saponins, and tannins. The study by Aryani et al. (2023) also revealed that flavonoids in *Ficus deltoidea* leaf extracts are strong reducing agents, suggesting that silver nanoparticles synthesized with *Ficus deltoidea* may be produced through the reduction of silver nitrate. Their research emphasizes the advantages of using nanoparticles as a drug delivery system, which include high drug loading and protection, prevention of premature drug leakage, biocompatibility and biodegradability, efficient targeting and cellular uptake, and controlled or autonomous drug release (Intasa-ard & Birault, 2019). Building on these findings, the potential of *Ficus deltoidea* flavonoids to facilitate nanoparticle synthesis suggests that a similar approach could be explored for Miracle Berry Leaf Extract, allowing for the development of nanoparticle-based drug delivery systems that enhance the extract's anti-angiogenic effects (Aryani et al., 2023).

Plant secondary metabolites have been shown to inhibit the growth of tumors by disrupting tumor-promoting signaling pathways (Varghese et al., 2020). Secondary metabolites can treat illnesses on their own or in conjunction with other substances or metabolites. These combinations have been shown in numerous trials to improve the effectiveness of treating a condition. Numerous phytochemicals have demonstrated remarkable efficacy in combating deadly illnesses like cancer (Seca et al., 2018)

Saponin is one of the highly valued bioactive groups due to its diverse positive effects, making it promising (Oekenfuull, 2023). Specifically, it has been observed in the study of Khan (2021), that it has the ability to scavenge free radicals and activate certain human embryonic kidney cells to overexpress antioxidant-related genes. Every section of a plant contains saponins and contains immunological and pharmacological qualities mostly studied for its anti-cancer properties. In contrary, presence of steroids in plants are relatively low and is recognized to possess antibacterial and cardiotonic activity. These secondary metabolites are frequently for medicinal purposes due to their impact on various biological activities (Vergara et al., 2021). The presence of quaternary ammonium surfactants or chemical compounds, due to their various chemical structure and

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biological capabilities, can be employed in medicine as DNA carriers, disinfectants, and antibacterial and anticancer agents. These chemicals caused the commencement of the apoptotic process of programmed cell death (Hyla et al., 2023)

Duck chorioallantoic membrane (CAM) Assay

The antiangiogenic properties of leaf crude extract of *Synsepalum dulcificum* was evaluated using the CAM assay. An increased blood vessel count at the treatment site on the CAM indicates angiogenic activity, whereas a reduced count suggests anti-angiogenic activity or inhibition of angiogenesis (Vergara et al., 2021).

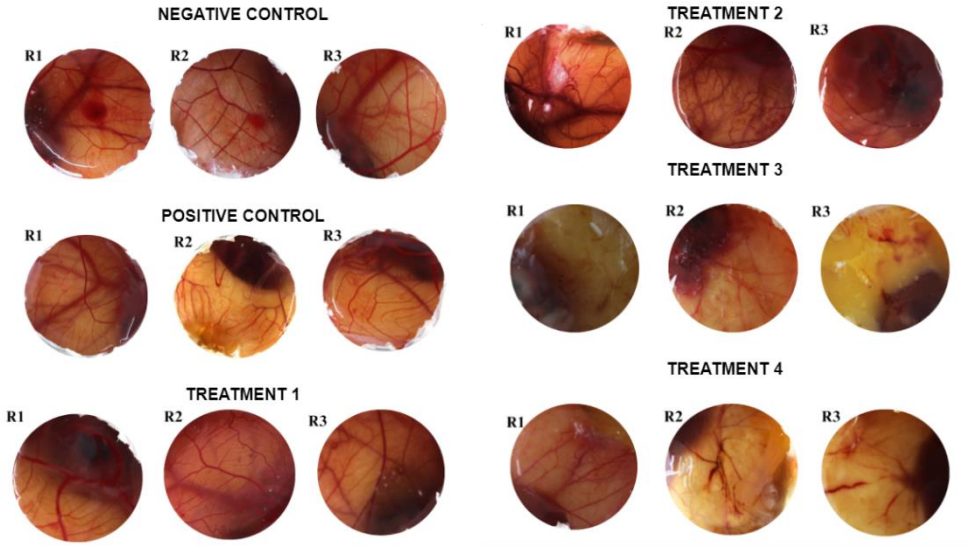

Figure 11. Duck CAM treated with distilled water (Negative Control), Retinoic Acid or Vitamin A (Positive Control), treated with Miracle Berry (*Synsepalum dulcificum*) Leaf Crude extract: 100 mg/L, 1,000 mg/L, 5,000 mg/L, 10,000 mg/L

Table 4. Vascular Density of the different treatments

Treatments	**Mean**	**Standard Deviation**
(PC) Vitamin A	169 ± 31.0	53.7
(T1) 100 mg mL^{-1}	125 ± 33.4	57.8
(T2) 1000 mg mL^{-1}	127 ± 57.3	99.2
(T3) 5000 mg mL^{-1}	56.0 ± 16.2	28.0
(T4) 10000 mg mL^{-1}	68.0 ± 13.0	22.6

As seen on Table 4, the Vitamin A or the positive control (PC) manifested the greatest number of vascular density (169 ± 31.0) among all groups. Similarly, all groups treated with *S. dulcificum* Leaf Crude Extract showed smaller vascular densities compared to the Vitamin A (169 ± 31.0). Among these, Treatment 3 (T3) with a concentration of 5,000 mg/L had the least vascular density (56.0 ± 16.2).

Known for its antiangiogenic properties, Vitamin A's ability to inhibit angiogenesis has been documented in various studies (Yang et al., 2022; Choi et al., 2024). It can modify the immune system, inhibit blood vessel formation, and regulate cell differentiation and growth. The lower vascular densities observed in these treatments, compared to the positive control**,** indicate the presence of antiangiogenic properties in the *S. dulcificum* Leaf Crude Extract. With this said, all groups treated with the *S. dulcificum* Leaf Crude Extract reveal lower vascular densities, thus the presence of anti-angiogenic activity.

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Table 5. One-Way ANOVA (Welch's) - Vascular Density

Measure	FFF	df1\text{df}_1df1	df2\text{df}_2df2	ppp
Vascular Density	5.31	5	5.45	0.039

One-Way Analysis of Variance (ANOVA) on the Vascular Density (Table 5) revealed that the vascular density of duck embryos treated with each concentration of *S. dulcificum* Leaf Crude Extract has a significant difference as its p-value is less than 0.05, thus we reject H04.

Table 6. Cohen's D (Effect Size) of the Four Treatments when compared to (PC) Vitamin A in terms of their Vascular Density

Treatments	Difference in Means	Pooled Standard Deviation	Cohen's D Effect Size	Interpretation
(T1) 100 mg mL^{-1}	44	55.79	0.79	Large Effect
(T2) 1000 mg mL^{-1}	42	79.76	0.53	Medium Effect
(T3) 5000 mg mL^{-1}	113	42.82	2.64	Large Effect
(T4) 10000 mg mL^{-1}	101	41.20	2.45	Large Effect

Interpretations: trivial effect *(d = .0 – 0.19)*, small effect (*d* = 0.2), medium effect (*d* = 0.5), large effect (*d* = ≥ 0.8)

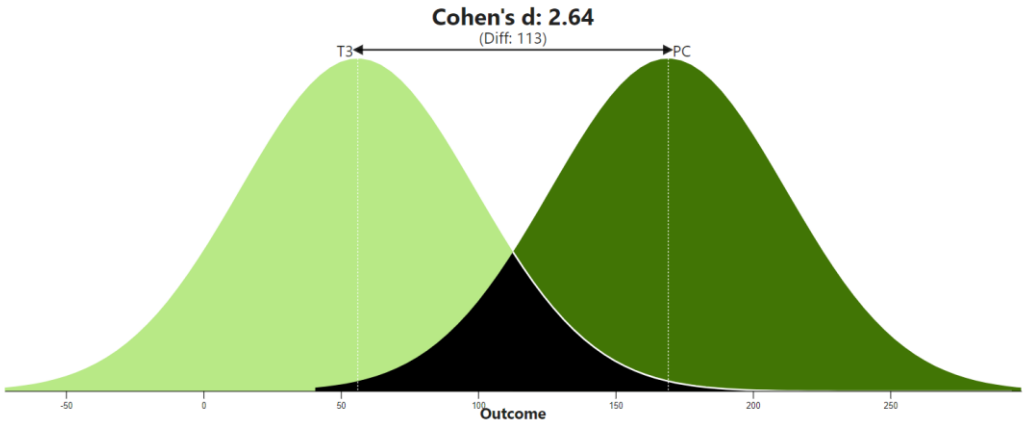

Figure 12. Cohen's D (Effect Size) Chart of (T3) 5000 mg mL^{-1} when compared to (PC) Vitamin A in terms of its Vascular Density

The Cohen's D or effect size of each treatment's vascular density when compared to Vitamin A revealed that whether or not there is a statistical difference between each of all the four treatments and Vitamin A, the findings indicate that Treatment 1, Treatments 3 and 4 exhibited Large effects, with effect sizes of 0.79, 2.64 and 2.45, respectively, all exceeding the standard deviation threshold of 0.8. Conversely, Treatment 2 showed a Medium effect size of 0.53. These results highlight the significant variations in effect sizes among the treatments relative to Vitamin A, and among the four treatments, Treatment 3 had the largest effect size.

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As previously mentioned, Treatment 3 (T3) at a concentration of 5000 mg/mL exhibits a Cohen's d of 2.64, the highest among the four treatments. This indicates that 99.6% of the samples from the T3 will exceed the mean of the samples treated with Vitamin A. As illustrated in Figure 12, there is an 18.7% overlap between the two groups. Furthermore, there is a 96.9% probability that a randomly selected sample from T3 will have a higher score than a randomly selected sample from the samples treated with Vitamin A (probability of superiority). Additionally, to achieve one more favorable outcomes in T3 compared to the samples treated with Vitamin A, an average of 1.3 samples are need to be treated. Therefore, if each group consists of 100 samples and it is assumed that 20 samples in the Vitamin A group exhibit favorable outcomes, then 3.0 + 3.0 samples in the T3 group are expected to exhibit favorable outcomes.

Table 7. Vascular Inhibition of Miracle Berry Crude Leaf Extract on different treatments

Treatments	**Mean**	**Standard Deviation**
(PC) Vitamin A	24.8 ± 6.87	11.9
(T1) 100 mg mL^{-1}	46.4 ± 7.27	12.6
(T2) 1000 mg mL^{-1}	50.5 ± 14.6	25.3
(T3) 5000 mg mL^{-1}	71.8 ± 8.14	14.1
(T4) 10000 mg mL^{-1}	65.7 ± 6.85	11.4

Among the four treatments, the highest level of inhibition was observed in eggs treated with 5,000 mg/L (71.8 ± 8.14) of the *S. dulcificum* Leaf Crude Extract followed by 1,000 mg/L (65.7 ± 6.580), 1000 mg/L (50.5 ± 14.6), and 100 mg/L (46.4 ± 7.27) respectively. Interestingly, T3 (5,000 mg/L) displayed the greatest level of inhibition in the study, even compared to the highest concentration of 10,000 mg/L applied on CAM. Additionally, the results shown on Table 7 also manifests a notable difference among the results of the groups treated with the *S. dulcificum* Leaf Crude Extract as compared to the Positive Control (24.8 ± 6.87).

The treatment groups exhibited a concentration-dependent response for vascular inhibition, with progressively increasing inhibition observed at higher concentrations of the *S. dulcificum* Leaf Crude Extract. This trend aligns with previous research by Palarca et al. (2022), who reported a concentration-dependent effect on angiogenesis and a decline in inhibition at the highest concentration for *Hyptis capitata*. Furthermore, Othman et al. (2019) conducted a study on *Morinda citrifolia* L. extract and found that it reduced blood vessel formation in a concentration-dependent manner. The group treated with a 75% concentration of the extract demonstrated a 37.1% reduction in blood vessels, whereas the groups with 50% and 25% concentrations showed lower reductions of 4% and 12.8%, respectively. This suggests that higher concentrations of *Morinda citrifolia* extract enhance anti-angiogenic effects, similar to the dose-response patterns observed with Miracle Berry Leaf Extract in this study. This phenomenon may be explained by mechanisms such as the suppression of vascular endothelial growth factor (VEGF) expression, matrix metalloproteinases (MMPs), and the inhibition of endothelial cell migration and proliferation, as reported by Subbaraj et al. (2021) for other anti-angiogenic agents.

Table 8. One-Way ANOVA (Welch's) - Vascular Inhibition

Measure	**FFF**	**df1\text{df}_1df1**	**df2\text{df}_2df2**	**ppp**
Vascular Inhibition	4.72	4	4.95	0.06

One-way analysis of variance (ANOVA) was performed to assess differences in vascular inhibition among treatment groups (Table 8). The results revealed non-significant p-values ($p > 0.05$), indicating that overall, there were no statistically significant differences in vascular inhibition between the groups. Based on this

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finding, the null hypothesis (H03) stating no difference in vascular inhibition across treatments could not be rejected. However, to further explore potential pairwise differences, a post-hoc test (Table 9) was employed. The post-hoc test revealed a significant difference (p-value < 0.05) between the control group and Treatment 3 (T3). This suggests that T3 induced the most pronounced changes in vascular inhibition compared to the control and potentially other treatment groups.

Table 9. Tukey Post-Hoc Test – Vascular Inhibition

Group Comparison	(PC) Positive Control	(T1) 100 mg mL−1^{-1}−1	(T2) 1000 mg mL−1^{-1}−1	(T3) 5000 mg mL−1^{-1}−1	(T4) 10000 mg mL−1^{-1}−1
Mean Difference	—	-21.6	-25.71	-47.0*	-40.95
p-value	—	0.495	0.341	0.03	0.062
Mean Difference	—	—	-4.11	-25.4	-19.35
p-value	—	—	0.997	0.352	0.591
Mean Difference	—	—	—	-21.3	-15.24
p-value	—	—	—	0.509	0.766
Mean Difference	—	—	—	—	6.05
p-value	—	—	—	—	0.989

As shown on the Tukey post-hoc test, Treatment 3 showed the highest efficacy among all four treatments, and even a higher efficacy compared to the maximum concentration of 10000 mg/L ppm applied on the CAM.

The CAM result aligns with Palarca et al. (2022), indicating a concentration-dependent impact on angiogenesis. Moreover, this may be influenced by the presence of the phytochemicals such as saponins and steroids. According to the findings of the study conducted by Mohammad Bagher Majnooni et al. (2023), the researchers found that saponins inhibit the expression of genes involved in VEGF release and activation, such as PI3K/mTOR/Akt, HIF-1α, bFGF, JAK/STAT, VEGFR2, and MAPK. They also directly impede the growth of vein endothelial cells. Studies have shown that saponins' chemical structure is directly related to their anti-angiogenic properties. Notably, triterpenoid and spirostanol saponins have cujstrong anti-angiogenic activities, making them interesting options for cancer therapy. Another positive phytochemical found in the leaf extract, a type of steroid- 2-deoxy sugars is a natural, non-metabolizable glucose analog that acts as a competitive glycolysis inhibitor by replacing the 2-hydroxyl group with hydrogen. 2-DG inhibits the activity of many glycolysis enzymes, resulting in cell death. Hyperglycemia exacerbates cancer cell proliferation, inflammatory diseases, and viral infections (Singh et al., 2023).

Table 10. Cohen's D Effect Size of the Four Treatments when compared to (PC) Vitamin A in terms of their Vascular Inhibition

Treatments	**Difference in Means**	**Pooled Standard Deviation**	**Cohen's D Effect Size**	**Interpretation**
(T1) 100 mg mL^{-1}	21.6	12.26	1.76	Large Effect

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(T2) 1000 mg mL^{-1}	25.7	19.77	1.30	Large Effect
(T3) 5000 mg mL^{-1}	47	13.05	3.60	Large Effect
(T4) 10000 mg mL^{-1}	40.9	11.65	3.51	Large Effect
Interpretations: trivial effect *(d = .0 – 0.19),* small effect (*d* = 0.2), medium effect (*d* = 0.5), large effect (*d* = ≥ 0.8)				

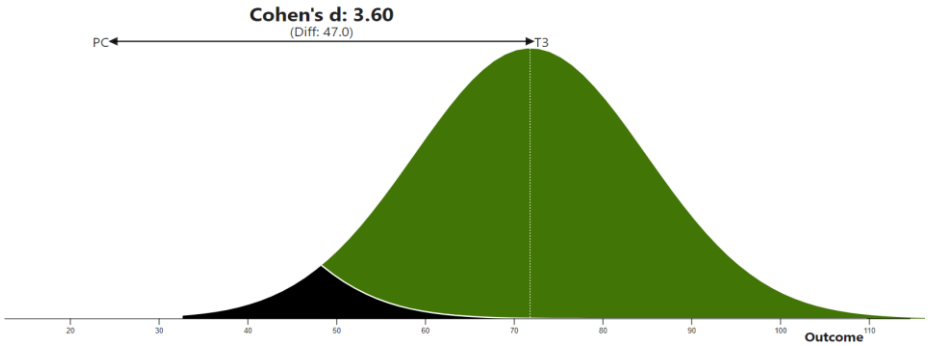

Figure 13. Cohen's D (Effect Size) Chart of (T3) 5000 mg mL^{-1} when compared to (PC) Vitamin A in terms of its Vascular Inhibition

The results of the Cohen's D effect size for vascular inhibition, comparing each of the four treatments to Vitamin A, indicate that all treatments had a large effect, as all effect sizes exceeded the standard deviation threshold of 0.8. Treatment 3 demonstrated the highest effect size with a standard deviation of 3.60, which indicates that its mean differ by 3.60 pooled standard deviations or its mean difference is greater than thrice the variability. This is followed by Treatment 4 with a standard deviation of 3.51 and indicates that its mean differs by 3.51 pooled standard deviations or a z-score of 3.5 from the mean of Vitamin A. Treatment 1 and Treatment 2 also showed substantial effects, with standard deviations of 1.76 and 1.30, respectively. These findings highlight the significant impact of each treatment in comparison to Vitamin A.

With a Cohen's d of 3.60 from T3, it is expected that 100% of the samples will surpass the mean of the samples treated with Vitamin A (PC). As illustrated in Figure 13, there is a 7.2% overlap between the two groups, and with the given data, there is a 99.5% probability that a randomly selected sample from T3 will have a higher score than a randomly selected sample from the samples treated with Vitamin A (probability of superiority). Furthermore, to achieve one additional favorable outcome in T3 compared to the Vitamin A treated samples, an average of 1.1 samples must be treated. This implies that if there are 100 samples in each group, and assuming that 3.0 samples from the Vitamin A treated group have favorable outcomes, then 3.0 + 92.7 samples in T3 will exhibit favorable outcomes.

Table 11. One-Way ANOVA (Welch's) - Vascular Inhibition and Vascular Density of All Four Treatments

Measure	FFF	df1\text{df}_1df1	df2\text{df}_2df2	ppp
Vascular Density	1.13	3	4.35	0.433
Vascular Inhibition	1.73	3	4.35	0.289

To evaluate the significant differences in vascular inhibition and vascular density among the four treatment groups, a One-Way ANOVA was performed (Table 11). The results indicated no statistically significant

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differences in either vascular density or vascular inhibition, as both p-values were greater than 0.05. This implies that varying concentrations exhibit the same inhibitory effect. Consequently, we fail to reject the null hypothesis (H01), which asserts that there are no significant differences in vascular inhibition and vascular density in the Chorioallantoic Membrane (CAM) Assay across the four concentration levels.

These findings hold promise for the development of novel anti-angiogenic agents derived from natural sources. The observed inhibition of vascularization aligns with prior research demonstrating the potential of plant extracts to impede tumor growth and metastasis (Palarca et al. 2022).

Morphometric Analysis

The morphometric analysis was done to determine the effect of *S. dulcificum* leaf extract on the growth development of the duck egg embryos and to determine the mean growth of the duck egg embryo at four concentrations. The embryo weight (EW), Crown-Rump Length (CRL), Head-Beak Length (HBL), Forelimb Length (FL), and Hindlimb Length (HL) were expressed in mean, with centimeter (cm) as the measurement unit.

The morphometric data, as shown in Appendix B, are incomplete due to some of the duck egg embryos' morphometrics being immeasurable as they are underdeveloped, as explained in page 128. Therefore, the incomplete data cannot be normally distributed and does not fit the assumption of normality and homogeneity which are the two major requirements in the performance of ANOVA. According to Verma and Abdel-Salam (2019), when these two requirements are violated, ANOVA should not be performed as this will lead to issues in internal validity. However, the Kruskal Wallis Test, a non-parametric alternative to the one-way ANOVA, is less sensitive to these assumptions. According to Nwobi and Akanno (2021), Kruskal Wallis is a more reliable method when the distributional assumptions of data sets are non-standard and skewed. The morphometric data gathered meets this test's assumptions of being non-normal or having a skewed distribution and having two or more groups that are not dependent on each other. Therefore, the Kruskal Wallis Test was performed to assess the mean growth and significant difference of duck embryos at four concentrations, as well as the significant difference in the mean growth of duck embryos between the four concentrations of Miracle Berry Leaf Crude Extract and Vitamin A.

Objective 6. Table 12. Kruskal-Wallis Test - Mean growth of duck egg embryos at four concentrations.

Morphometry						
Treatment	**EW (g)**	**CRL (cm)**	**HBL (cm)**	**FLL (cm)**	**HLL (cm)**	**ED (cm)**
(T1) 100 mg mL^{-1}	1.23 ± 0.0333	29.3 ± 0.333	11.7 ± 0.664	4.67 ± 0.333	11.0 ± 0.577	1.67 ± 0.333
(T2) 1000 mg mL^{-1}	1.10 ± 0.115	29.7 ± 0.883	11.0	3.00	11.0 ± 0.999	2.00
(T3) 5000 mg mL^{-1}	0.667 ± 0.167	28.0	11.0	4.00	9.00	1.00
(T4) 10000 mg mL^{-1}	0.733 ± 0.233	29.0 ± 0.814	11.5 ± 0.408	4.50 ± 0.408	6.00 ± 4.08	1.00

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Figure 14. Underdeveloped Eggs in Treatments 3 and 4

As shown in Table 12, the lowest concentration of Miracle Berry Leaf Crude Extract at 100 mg/L showed the highest mean embryo weight (1.23 ± 0.0333 g) and relatively high values in other morphometric parameters: CRL (29.3 ± 0.333 cm), HBL (11.7 ± 0.664 cm), FLL (4.67 ± 0.333 cm), HLL (11.0 ± 0.577 cm), and ED (1.67 ± 0.333 cm). At a higher concentration of 1000 mg/L, there was a slight reduction in mean weight (1.10 ± 0.115 g) and CRL (29.7 ± 0.883 cm), with HBL (11.0 cm) remaining constant. A significant reduction in FLL to 3.00 cm was observed, while HLL (11.0 ± 0.999 cm) and ED (2.00 cm) showed minimal changes. At 5000 mg/L, the mean weight of the embryos dropped significantly to 0.667 ± 0.167 g, with CRL reducing to 28.0 cm. Despite HBL remaining constant at 11.0 cm, there was a decrease in FLL to 4.00 cm, HLL to 9.00 cm, and ED to 1.00 cm. The highest concentration of 10000 mg/L resulted in a mean weight increase to 0.733 ± 0.233 g compared to 5000 mg/L but still lower than the 100 mg/L and 1000 mg/L groups. The CRL was 29.0 ± 0.814 cm, HBL was 11.5 ± 0.408 cm, FLL was 4.50 ± 0.408 cm, HLL dropped sharply to 6.00 ± 4.08 cm, and ED was 1.00 cm. To conclude, the morphometric measurements of duck egg embryos at the 100 mg/L concentration show the highest values across almost all parameters, while the 5000 mg/L and 10000 mg/L concentrations have lower values.

The results clearly show that the higher the concentration, the greater the reduction in the morphometric measurements of duck embryos. This observation is consistent with the study of Pastor and Almadin (2017), which documented that the values generally decrease as the concentration increases. They observed a significant reduction in CAM vasculature with higher concentrations of *C. cujete* extract but also noted increased sample mortality. This suggests that optimizing concentration levels might be crucial to maximizing anti-angiogenic effects while minimizing toxicity. A large number of observable underdeveloped eggs were noted in their work. Since the *S. dulcificum* extract was found to have certain secondary metabolites, excessive accumulation may have caused this similar finding. This is corroborated in the studies of Gonzales (2022); Leon (2012) which explained that phytochemicals display anti-angiogenic properties and having underdeveloped morphology compared to the untreated duck embryos is not surprising as they could also be destructive to a certain level. Furthermore, saponins are highly toxic when administered intravenously in animals (Leon, 2018), and considering the treatment was applied in the duck embryos' CAM, which is highly vascularized, this may also be one of the explanations. Despite Treatment 4 being the highest concentration in this study, the extract is most toxic to embryonic development in Treatment 3, as evidenced by the observed underdeveloped morphology shown in Figure 14 when statistically compared to other concentrations. Initially, eggs treated with 10,000 mg/L have bigger initial weights, in comparison with those that are exposed to 5, 000 mg/L. Eggs in T4 and T3 have approximately a 10-gram difference. This observed trend might be attributable to inherent variations in initial egg weight and lack of randomization. Previous research by Hegab and Hanafy (2019) suggests that larger eggs exhibit significantly greater total pore count, surface area, eggshell volume, and embryo weight compared to smaller eggs. These inherent differences in starting egg weight could potentially influence embryo weight outcomes, even if the treatment itself does not directly affect growth.

Furthermore, it can be inferred that the result provides evidence for the anti-angiogenesis effect of Miracle Berry Leaf Crude extract through its impact on embryonic development, specifically by demonstrating toxicity at higher concentrations which likely disrupts angiogenesis. Angiogenesis, the formation of new blood vessels, is a critical process in embryonic development (Naito et al., 2020). The underdeveloped morphology observed

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in embryos exposed to 5000 mg/L and 10,000 mg/L concentrations indicates that the extract interferes with this process.

Objective 7. Table 13. Kruskal-Wallis Test - Significant difference in the mean growth of duck egg embryos at four concentrations.

Morphometry						
	EW (g)	CRL (cm)	HBL (cm)	FLL (cm)	HLL (cm)	ED (cm)
P-value	0.078	0.604	0.608	0.092	0.515	0.133

As presented in Table 13, statistical analysis (p-values) revealed no significant differences across treatments for embryo weight (p=0.078), CRL (p=0.604), HBL (p=0.608), HLL (p=0.515), and ED (p=0.133), and FLL (p=0.092) at four concentrations of Miracle Berry Leaf Crude extract. Therefore, the data does not support the rejection of the null hypothesis (H04).

This finding is similar with the results found in the studies of Vergara et al. (2021) and Palarca et al. (2022 where there were no significant differences found in the mean growth of the duck egg embryos in their treatments. The lack of significant differences suggests that all four concentrations may have similar anti-angiogenic effects. Despite the observed trends indicating lower morphometric values and underdeveloped embryos at higher concentrations, the statistical analysis showing no significant differences implies that the extract's impact on angiogenesis could be consistent across the tested concentrations. This consistency in anti-angiogenic effects across different concentrations might indicate that even the lower concentrations of Miracle Berry Leaf Crude extract are sufficient to disrupt angiogenesis to a degree similar to higher concentrations. Consequently, while higher concentrations exhibit more pronounced signs of toxicity and undeveloped eggs, the fundamental anti-angiogenic properties appear to be present at all experimental group concentrations.

Objective 8. Table 14. Kruskal-Wallis Test - Mean growth of duck egg embryos among the four concentrations of Miracle Berry Leaf Crude Extract and Vitamin A.

Morphometry						
Treatment	Weight (g)	CRL (cm)	HBL (cm)	FLL (cm)	HLL (cm)	ED (cm)
(PC) Vitamin A	1.47 ± 0.167	31.3 ± 0.883	12.3 ± 0.333	5.67 ± 0.333	12.0 ± 0.577	1.00
(T1) 100 mg mL^{-1}	1.23 ± 0.0333	29.3 ± 0.333	11.7 ± 0.664	4.67 ± 0.333	11.0 ± 0.577	1.67 ± 0.333
(T2) 1000 mg mL^{-1}	1.10 ± 0.115	29.7 ± 0.883	11.0	3.00	11.0 ± 0.999	2.00
(T3) 5000 mg mL^{-1}	0.667 ± 0.167	28.0	11.0	4.00	9.00	1.00

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(T4) 10000 mg mL^{-1}	0.733 ± 0.233	29.0 ± 0.814	11.5 ± 0.408	4.50 ± 0.408	6.00 ± 4.08	1.00
P-value	0.033	0.249	0.222	0.053	0.366	0.075

As presented in Table 14, Vitamin A, used as a positive control, resulted in the highest mean weight (1.47 ± 0.167 g) and longest Crown-Rump Length (CRL) of 31.3 ± 0.883 cm. Other morphometric measurements in this group were also the highest in: Head-Beak Length (HBL) at 12.3 ± 0.333 cm, Forelimb Length (FLL) at 5.67 ± 0.333 cm, Hindlimb Length (HLL) at 12.0 ± 0.577 cm, and second highest in Eye Diameter (ED) at 1.00 cm among all concentrations. At a concentration of 100 mg/L, the mean weight of the embryos decreased to 1.23 ± 0.0333 g, with a reduction in CRL to 29.3 ± 0.333 cm. HBL also decreased to 11.7 ± 0.664 cm, and FLL to 4.67 ± 0.333 cm. HLL dropped to 11.0 ± 0.577 cm, while ED increased to 1.67 ± 0.333 cm. Increasing the concentration to 1000 mg/L resulted in a further decrease in mean weight to 1.10 ± 0.115 g. The CRL was slightly higher than the 100 mg/L group at 29.7 ± 0.883 cm. However, the HBL remained constant at 11.0 cm, and the FLL significantly reduced to 3.00 cm. HLL showed minimal change at 11.0 ± 0.999 cm, and ED increased to 2.00 cm. At the higher concentration of 5000 mg/L, there was a significant reduction in mean weight to 0.667 ± 0.167 g, and CRL dropped to 28.0 cm, reflecting a substantial impact on overall embryonic growth. Despite the HBL remaining at 11.0 cm, FLL was observed at 4.00 cm, and HLL reduced to 9.00 cm. The ED was reduced to 1.00 cm. The highest concentration tested, 10000 mg/L, showed a slight increase in mean weight to 0.733 ± 0.233 g compared to the 5000 mg/L group, but it remained lower than the weights observed at 100 mg/L and 1000 mg/L. The CRL was 29.0 ± 0.814 cm and HBL was increased slightly to 11.5 ± 0.408 cm, and FLL to 4.50 ± 0.408 cm. Also, HLL dropped sharply to 6.00 ± 4.08 cm and ED remained constant at 1.00 cm. Statistical analysis revealed no significant difference for most parameters (p-values: weight 0.033, CRL 0.249, HBL 0.222, FLL 0.053, HLL 0.366, ED 0.075).

Based on the statistical analysis, the embryo weight parameter is the only one that shows a statistically significant difference, with a p-value of 0.033. This finding indicates that the different concentrations of Miracle Berry Leaf Crude Extract and Vitamin A have a significant effect on the weight of the embryos. Except for embryo weight, morphometric parameters such as Crown-Rump Length (CRL), Head-Beak Length (HBL), Forelimb Length (FLL), Hindlimb Length (HLL), and Eye Diameter (ED) with p-values (0.249, 0.222, 0.053, 0.366, and 0.075 respectively) suggest that there are no statistically significant differences across the four concentrations and Vitamin A. Therefore, the null hypothesis (H05), which posits that there is no significant difference in the mean growth across the experimental group and positive control, cannot be rejected. It can also be inferred that the anti-angiogenic effects of Vitamin A and Miracle Berry Leaf Crude Extract are similar when considering the parameters of CRL, HBL, FLL, HLL, and ED. Despite the different concentrations of Miracle Berry Leaf Crude Extract used, their effects on the overall growth and development of duck embryos on these parameters are indistinguishable from those observed with Vitamin A. Therefore, the results indicate that the leaf extract, even at varying concentrations, does not affect these specific morphometric parameters differently compared to Vitamin A.

CONCLUSION

The research clearly shows that Miracle Berry Leaf Crude Extract *(Synsepalum dulcificum)* possesses strong anti-angiogenic characteristics that efficiently impede the growth of new blood vessels. Reduced vascular densities, which were reported at all tested concentrations, are indicative of the extract's strong anti-angiogenic activity. Furthermore, it may be deduced that there is an increase in vascular inhibition in proportion to the leaf extract concentration. The study also revealed that there were no statistically significant differences between the treatments in vascular inhibition. This finding indicates that, at varying concentrations, the extract can consistently inhibit vascular growth. The morphometric analysis also showed nonsignificant differences between the treatments. This supports the conclusion that Miracle Berry Leaf Crude Extract and Vitamin A, which is a known anti-angiogenic substance, exhibit comparable anti-angiogenic effects, particularly in terms of the structural development of embryos. This implies that Miracle Berry Leaf Crude Extract might have comparable medicinal advantages, but it should be carefully considered that it may be teratogenic since higher

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concentrations cause obvious underdevelopment or toxicity effects in embryos, indicating a direct correlation between concentration and toxicity. Overall, the study highlights the extract's promising anti-angiogenic potential and points to the need for more investigation to completely grasp its therapeutic uses and weigh the advantages against the disadvantages. How to efficiently utilize the extract's anti-angiogenic properties while reducing any toxic effects could be the subject of future research.

RECOMMENDATIONS

Department of Health (DOH). Recommend to expand public access to safe and effective herbal remedies and drive the development of healthcare policies grounded in rigorous, evidence-based research. Such policies would ultimately foster a healthcare system characterized by greater effectiveness and efficiency since aligning with the DOH's list of 10 approved herbal medicines, the findings from this research can contribute valuable insights that may lead to the inclusion of new medicinal plants, such as the Miracle Berry, in this esteemed list.

Future Researchers. Recommend this research to those who plan to have the same line of topic or would like to know more regarding this study. Furthermore, the researchers recommend exploring two or more different methodologies in phytochemical analysis to achieve more accurate results (Shaikh & Patil, 2020). This is because phytochemical analysis, which provides a foundation for the targeted isolation of compounds and enables more precise investigations, largely depends on the solvent used for extraction. This approach accounts for variations in methodologies used in different phytochemical screenings conducted across studies. Furthermore, the researchers suggest conducting additional investigations to isolate the specific components responsible for the observed teratogenic and toxic effects. The inhibition of blood vessel growth in the study may be linked to certain phytochemicals in the leaf extract, including saponins, steroids, and quaternary bases and amine oxides (Magdadaro, 2023). Given that the crude leaf extract exhibits high toxicity at elevated concentrations—evidenced by underdeveloped embryonic morphology—future studies should explore the effects of lower extract concentrations or incorporate toxicity tests. This approach will help prevent structural discrepancies in model organisms, as discussed by Pastor & Almadin (2017). The researchers also emphasize the importance of randomizing egg distribution to mitigate bias. Studies by Sarogni et al. (2022) and Rupp et al. (2022) effectively utilized randomization in the CAM Assay, distributing eggs into groups to ensure even distribution of inherent variations, such as initial egg weight. This practice minimizes the risk of bias that could influence experimental outcomes. Randomly assigning eggs ensures that each treatment group starts on a comparable basis, making it more likely that any observed differences are attributable to the treatments rather than pre-existing disparities. Fowler & Fleming (2023) underscore that successful randomization is a crucial research technique, as it eliminates potential personal bias and extraneous variables that could distort the results. Additionally, the researchers strongly recommend maintaining consistent initial duck egg weights across all groups. Hegab and Hanafy (2019) noted that larger eggs tend to produce larger embryos, so significant differences in initial egg weights between groups could lead to disparities in results. To address this, efforts should be made to standardize egg sizes or account for weight differences in the analysis. Lastly, the researchers advise furthering the examination of Miracle Berry Leaf Extract by sending it to specialized laboratories for testing on actual cancer cells, given the study's demonstration of the extract's anti-angiogenic qualities. Comprehending this stage is essential for evaluating its possible therapeutic efficacy and comprehending its mode of action.

Academic Institution. This study might inspire Father Saturnino Urios University to create its own Institutional Animal Care and Use Committee (IACUC). This advancement would make it easier to conduct such experiments in the future and give students excellent chances to participate in real-world animal experimentation. The university's commitment to expanding scientific understanding while upholding the highest standards of animal welfare would be highlighted by the establishment of an IACUC. By attracting committed staff and students, this effort would establish the university as a leader in ethical research methods. In the end, it might result in ground-breaking discoveries and inventions that benefit society as a whole as well as the academic community.

Local Government Unit's Sangguniang Panlungsod - Committee on Science and Technology. Recommend to consider this study's findings in the hope that these results will not only bolster support for

antiangiogenic therapy but also stimulate further research into its health benefits. As the field of angiogenesis research continues to expand, the insights from this study could play a pivotal role in advancing the city's commitment to fostering technological and research advancements. By showcasing the potential of the Miracle Berry Leaves, this research may encourage local leaders to invest more in cutting-edge scientific endeavors. Such investment could pave the way for exploring innovative ideas, enhancing the city's reputation as a hub of medical and technological innovation, and creating a dynamic scientific community that benefits both researchers and the broader public.

Local Government Unit's Sangguniang Panlungsod - Committee on Health and Sanitation. Suggest that they consider this study since the findings could spark greater interest from the local government in exploring medicinal plants, such as the Miracle Berry, which are readily available in the city and may hold key compounds contributing to effective cancer treatments. By focusing on locally sourced, natural remedies, the government could support both scientific advancement and the utilization of regional resources. This approach not only fosters a sense of community but also positions the city as a leader in innovative and sustainable healthcare solutions. Encouraging investment in this area may lead to significant breakthroughs, benefiting the health and well-being of Butuanons and setting a precedent for other regions to follow. The researchers believe that the effects of the Miracle Berry Leaves could serve as a starting point for further exploration, both domestically and internationally, in the field of antiangiogenic therapy and its health benefits.

Department of Science and Technology (DOST). The researchers strongly recommend that the DOST take this study into consideration. By advancing the understanding of angiogenesis, a fundamental biological process essential for growth, development, and wound healing, through studies like this, we can develop more effective antiangiogenic therapies that inhibit tumor growth and spread. Such research is crucial for the progression of cancer treatment, as it offers new strategies to combat the disease by cutting off the blood supply to tumors. This could lead to developments in oncology, offering new hope for patients and significantly contributing to the global fight against cancer.

Health Care Professionals. Researchers highly recommended this study to the healthcare professionals, particularly those in the healthcare and public health sectors. By delving deeper into this research, healthcare workers can enhance their knowledge of medicine and potentially expand their understanding of the Miracle Berry Leaf's impact as an anti-angiogenic agent. It is recommended that healthcare professionals consider using this plant as a complementary or alternative treatment in the management of angiogenesis-related conditions, including cancer. Additionally, they should take an active role in educating patients and the community about the potential benefits of the Miracle Berry while also spreading awareness about the possible teratogenic effects of the leaf. This balanced approach ensures that while exploring the therapeutic potentials, necessary precautions are taken to avoid any teratogenic effects, thereby fostering a more informed and safer use of herbal medicine in clinical practice especially for pregnant women.

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