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Anti-Angiogenesis Effects of Citrus and Flaxseed Extracts in a Chorioallantoic Membrane Assay

Raymund J. Capagas*, Mary Jobhel Callueng, Daschel Alliyah Diega, Aaliyah Dominique Gonzales, Benedict Isleta, Dhana San Agustin
National University – Manila, Metro Manila, Philippines

*Corresponding Author Email: rjcapagas@nu-moa.edu.ph

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Abstract. Both *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed) are rich sources of bioactive compounds with potential health benefits, including anti-cancer properties. This study assessed and compared the overall anti-angiogenesis activity of *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed). Specifically, this study aimed to determine the minimum concentration of both *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed) that exhibits anti-angiogenesis activity. It also aimed to determine the most potent concentration that is effective as an anti-angiogenesis agent. This study investigated the anti-angiogenic activity of *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed) ethanolic extract using the fertilized duck embryo chorioallantoic membrane (CAM) assay, a widely accepted method for studying angiogenesis. Several concentrations of *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed) ethanolic extracts were applied to the CAM of duck embryos, while control groups received saline and ascorbic acid. Preliminary findings suggest that *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed) ethanolic extracts exhibit anti-angiogenesis activity. Further analysis, including quantifying blood vessel density and branching, provided insights into the comparative efficacy of the two extracts. Statistical analysis revealed that the most effective ethanolic concentration for *Citrus aurantium* (Dalandan) is 10%, while for *Linum flavum* (Golden Flaxseed) is 30%. The study concluded that *Citrus aurantium* (Dalandan) has the most effective anti-angiogenic activity compared to *Linum flavum* (Golden Flaxseed). This study contributes to understanding the potential use of *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed) for therapeutic applications in cancer treatment through their anti-angiogenic properties. Moreover, this study underscores the importance of utilizing alternative models, such as the CAM assay, for rapid and cost-effective screening of anti-angiogenic agents.

Keywords: Anti-angiogenesis, *Citrus aurantium*; Duck embryo CAM assay; *Linum flavum*.

1.0 Introduction

Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels. This process involves the migration, growth, and differentiation of endothelial cells, which line the inside wall of blood vessels (Cleveland Clinic, 2022). Angiogenesis is the process of blood vessel formation from pre-existing ones, mainly observable during the development of embryos and fetuses. However, it can also be observed in adults during wound healing and other physiological mechanisms (Teleanu et al., 2019). Angiogenesis plays a crucial role in the growth and spread of cancer. Tumors require a blood supply to receive nutrients and oxygen, and angiogenesis provides the means. Angiogenesis is essential for tumors to get oxygen and nutrients and help them spread (Teleanu et al., 2019).

In an article published by the World Health Organization, studies identified that cancer was responsible for nearly 10 million deaths in 2020, and studies projected that the number of new cancer cases will increase by about 70% over the next two decades. In 2020 alone, there were an estimated 19.3 million new cancer cases worldwide, with several types, such as breast, lung, colorectal, and prostate cancer, ranking among the most diagnosed forms of the disease (Ferlay, 2021). That said, plants contain various active chemical compounds, which can synergistically affect the body. It means it simultaneously influences the different aspects of a disease's pathology. For instance, plant extracts rich in biologically active compounds can slow down the growth of cancer cells and induce apoptosis in them simultaneously, leading to tumor eradication by hindering angiogenesis and, consequently, metastasis (Khan et al., 2022).

In a 2018 study by Mannucci et al., *Citrus aurantium* was known for its unique phytochemical profile and extensive health benefits, with high concentrations of flavonoids, limonoids, and alkaloids (Mannucci et al., 2018). Flavonoids, especially rutin, constitute a substantial portion, and limonoids and alkaloids in the plant have demonstrated anti-cancer, antiproliferative, cardioprotective effects, and hypolipidemic. On the other hand, oil extracted from Golden Flaxseed (*Linum flavum*) contains flavonoids, which show its potential to inhibit the formation of new blood vessels, showing its anti-cancerous and antioxidant activities (Al-Zubaidy & Sahib, 2022). Moreover, a study by (Chera, 2022) identified that *Linum flavum* (Golden Flaxseed) can undergo ethanolic extract. The discovery of the plant's anti-tumor, antioxidant, and anti-inflammatory potential was possible through the ethanolic extraction of the derivatives of *Linum flavum* (Golden Flaxseed). The study's findings showed the antitumoral, antioxidant, and anti-inflammatory qualities of Golden Flaxseed-derived ethanol extract.

Due to the promising potential of both *Citrus aurantium* (Dalandan) and the *Linum flavum* (Golden Flaxseed) for anti-angiogenesis activity and because there is a need to conduct more research about the natural alternative to help fight cancer, this study aims to assess further the efficacy and potency of both peels of *Citrus aurantium* (Dalandan) and the *Linum flavum* (Golden Flaxseed) ethanolic extract as anti-angiogenesis agent. This study also aims to compare which among the two test plants exhibits high potential to be an anti-angiogenesis agent using a C.A.M. Assay. In conducting the C.A.M. assay, four ethanolic concentrations per plant were prepared, including positive and negative controls. To monitor morphological changes and inhibit blood vessel growth, fertilized duck eggs were precisely selected and thoroughly cleansed. The study purchased duck eggs from a licensed duck embryonic egg (fertilized native duck egg) raiser near the country. This study assessed the anti-angiogenic activity of *Citrus aurantium* and *Linum flavum* ethanolic extracts using the Duck Embryo Chorioallantoic Membrane (C.A.M.) Assay. The analysis of the antioxidant properties and the anti-angiogenic activity aided the assessment of the two plants (Barluado et al., 2013).

Further, this study extracted peels of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) using ethanolic extractions. The study used duck eggs to assess the anti-angiogenic properties of the plants through the administration of a series of extract concentrations from 10%, 30%, 50%, and 70%, in which the study determined the effectiveness of the respective concentrations through the number of newly formed blood vessels exposure to the extract concentrations. Anticipating the study's outcomes, the study expected that *Citrus aurantium* and *Linum flavum* ethanolic extracts exhibited anti-angiogenic activity, substantiated by inhibiting blood vessel growth in the C.A.M. assay. The comparison of their efficacy at different concentrations revealed valuable insights into which plant extract demonstrates a superior anti-angiogenic activity. This study hypothesized that the phytochemical composition of *Citrus aurantium*, containing phenolic acids and flavonoids (Teleanu et al., 2019), and *Linum flavum*, with flavonoids (Al-Zubaidy & Sahib, 2022), contributed to their observed anti-angiogenic activities. The studies expected the results to provide quantitative data on the concentration at which each plant extract effectively inhibits angiogenesis, offering crucial information that can contribute to the scientific understanding of plant-derived compounds as potential agents in anti-angiogenic therapies for cancer and possible therapeutic applications.

2.0 Methodology

2.1 Research Design

The chosen research design for this study is an experimental comparative design, explicitly employing the Duck Embryo Chorioallantoic Membrane (C.A.M.) assay to investigate the comparative anti-angiogenesis activity of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) extracts. Since the study extensively elaborated on both plants' phytochemical components, phytochemical analysis was not conducted in this study. The research involved methodically preparing the extracts, exposing duck embryos to different concentrations, and measuring the blockage of blood vessels at specific intervals. Based on formulated hypotheses, the study aimed to compare the anti-angiogenic effects of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) extracts to determine their varying abilities to inhibit blood vessel formation. The examination of ethical concerns supported the utilization of duck embryos while applying statistical techniques to facilitate data analysis. The anticipated outcomes of this study are necessary to improve our understanding of comparative anti-angiogenic activities while considering limitations such as specific test settings and variability in extracts. The samples needed, specifically the *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed), were acquired from a direct-to-farm market known as Dizon Farms, a farm located in Sitio Zambal, Daranak Falls Rd. Tanay, Rizal. The researchers purchased the plants from Dizon Farms, a well-known local organic farm in various provinces in the Philippines, including Davao and Pampanga. Moreover, the subject for the C.A.M. Assay, the fertilized duck egg, was acquired from a licensed fertilized duck egg raiser located in Pateros, Metro Manila. The researchers acquired a fertilized egg on the first day of its incubation period, and then the researchers continued the incubation until the fourteenth day of the incubation period.

2.2 Research Locale

The researchers conducted the study at the National University Mall of Asia, where the researchers utilized the fully equipped laboratory to carry out biological assays, including the Duck Embryo Chorioallantoic Membrane (C.A.M.) assay, which is central to this study, to assess the anti-angiogenic activity of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed). While the laboratory setting offered controlled conditions for conducting experiments, it is essential to acknowledge certain limitations. These include the inability to fully replicate the complex microenvironment of in vivo angiogenesis within the laboratory setting and potential variations in experimental outcomes due to biological factors inherent to the duck embryo model.

2.3 Statistical Analysis

The study utilized ImageJ Software to quantify the blood vessels of the duck embryo found on its chorioallantoic membrane (C.A.M.). In data analysis, descriptive statistics was used to present the mean of the formed blood vessels per study concentrations and positive control. A multiple regression analysis was utilized to compare the anti-angiogenic activity of both plants.

2.4 Data Gathering Procedure

The researchers used fertilized duck embryos to assess the anti-angiogenic activities of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) ethanolic extracts. The acquired fertilized duck eggs are one-day-old eggs. After obtaining the fertilized ducks from a licensed fertilized duck egg raiser, the researchers incubated them for up to 13 days more since most duck eggs require approximately 28 days to hatch. (Hatching Duck Eggs, n.d.). The fertilized duck eggs were incubated for at least 37C (Anglo-Ojeda, 2022).

The researchers acquired *Citrus aurantium* (Dalandan) from a direct-to-farm market known as Dizon Farms. The first step includes the removal of the fruit's skin to isolate what was only needed. The *researchers gathered the peels of Citrus aurantium* (Dalandan) into a tray and then dehydrated them for approximately 1 hour at 150c using a standard microwave oven. The researchers used a mortar and pestle to pulverize the dried *Citrus aurantium* (Dalandan). Upon dehydration, the peels were prepared in the next step using a 1000ml beaker. The researchers used ethanol to subject the pulverized form of the *Citrus aurantium* (Dalandan) peels to ethanolic extractions. To prepare the ethanolic extraction of *Citrus aurantium* (Dalandan) peels, a 1:10 ratio was applied. Next, the researchers transferred 180 ml of 70% ethanol to the beaker containing 20g of pulverized peels. This step marks the beginning of the maceration. The ethanolic solution was then magnetically stirred. A magnetic stirrer ensured the ethanolic solvent was even when mixing with the sample material. The magnetic stirrer helps maintain a constant interaction between the ethanol and the sample through continuous stirring of the mixture. This process can enhance the target compounds' solubilization and increase the extraction process's overall yield. The solution underwent magnetic stirring for 5 minutes at 90c. The solution was left to soak for 24 hours for the flavonoids to yield accordingly, wherein the substance was stored in a dark and dry area to ensure the quality of the solution.

After 24 hours of soaking, a stirring rod was utilized in the experiment to mix the solution to avoid clumping of sediments. Then, the researchers transferred the solution to multiple test tubes in preparation for the ethanolic water bath extraction. The solution's test tubes underwent water bath treatment for 30 minutes at 90c. After ethanolic water bath treatment, a viscous extract remained at the bottom of the test tubes. This viscous extract contains the targeted compounds that will exhibit anti-angiogenic activity. After ethanolic-water bath extraction, the test tubes were centrifuged for 3 minutes at 30 rpm to ensure that the viscous extract would be sedimented and isolated. The researchers then transferred viscous extract into a 1000ml beaker to prepare the stock solution. Implementing a 1:10 ratio, 20g of the extract was diluted with distilled water to derive the *Citrus aurantium* (Dalandan) stock solution. The next step included pouring the stock solution into smaller-sized 200ml beakers to prepare the respective dilutions.

Then, the researchers prepared a series of ethanolic extract concentrations varying from 10%, 30%, 50%, and 70%. Both plants undergo ethanolic extraction to have equal footing using a 70% ethanolic extraction. (Barluado et al., 2013), Ethanol extract for *Citrus macrocarpa* peels uses a ratio of 1:10, and per 0.10 g/ml of extract, 70% ethanol was used in the study to macerate the peels. Thus, the study implemented a 1:10 solvent ratio for *Citrus aurantium* (Dalandan).

The researchers utilized 20g of *Citrus aurantium* (Dalandan) peels and 180 ml of 70% ethanol as a solvent. Thus, the researchers yielded a total of 200 plant extracts. The stock solution was further diluted into four different concentrations to contextualize the concentration to the study's objectives. Below is the computation for each concentration:

Let,
V_s = Volume of stock solution
V_w = Volume of distilled water
V_t = Total Volume after dilution
v/v% = Volume Percent

The formula for the total Volume (V_t) can be expressed as the sum of the Volume of the stock solution (V_s) and the Volume of distilled water (V_w):

$V_t = V_s + V_w$

Table 1. Computation of dilution of the stock solution of *Citrus aurantium*

v/v%	Vs + Vw	Vt
For a 10% concentration	$10\%=\left(\frac{10}{100}\right)$ 200ml Vs+180mL of distilled water	200mL of 10% diluted stock solution
For a 30% concentration	$30\%=\left(\frac{30}{100}\right)$ 200ml Vs+140mL of distilled water	200mL of 30% diluted stock solution
For a 50% concentration	$50\%=\left(\frac{50}{100}\right)$ 200ml Vs+100mL of distilled water	200mL of 50% diluted stock solution
For a 70% concentration	$70\%=\left(\frac{70}{100}\right)$ 200ml Vs+60mL of distilled water	200mL of 70% diluted stock solution

Table 2 Dilution of the stock solution *Citrus aurantium*

v/v%	Stock Solution	Distilled Water (Diluent)
10% concentration	20ml	180ml
30% concentration	60ml	140ml
50% concentration	100ml	100ml
70% concentration	140ml	60ml

As shown in Tables 1 and Table 2, to dilute the stock solution to a 10% concentration, a portion equal to one-tenth of the original stock solution is combined with approximately 180 mL of distilled water. This mixture yields a final volume of 200 ml, achieving the desired 10% concentration. The dilution process is straightforward, adhering to the formula derived from the mass conservation principle.

Similarly, when aiming for a 30% concentration, three-tenths of the stock solution was diluted with 170mL of distilled water. The result is a 200 ml final volume of a 30% diluted stock solution. The researchers applied the same approach for concentrations of 50% and 70%, mixing five-tenths of the stock solution with approximately 100mL of distilled water and seven-tenths of the stock solution mixed with approximately 60mL of distilled water to maintain the final Volume at 200ml for each case. After dilution, the researchers used an amber bottle to store the total solution. This process demonstrates the consistency and adaptability of the dilution formula in achieving different concentration levels with precision.

The researchers acquired *Linum flavum* (Golden Flaxseed) from a direct-to-farm market known as Dizon Farms. The seeds did not undergo prior preparation compared to the *Citrus aurantium* peels. Hence, it was ready for dehydration. The seeds were dehydrated using a standard microwave oven for approximately 0.5 hours at 150c. The researchers used a mortar and pestle to pulverize the dried *Linum flavum* (Golden Flaxseed). Upon dehydration, they prepared the seeds in a 1000ml beaker. The researchers used ethanol to subject the pulverized form of the *Linum flavum* (Golden Flaxseed)—seeds for ethanolic extractions. The researchers applied a 1:10 ratio to prepare the ethanolic extraction of seeds. Next, the researchers transferred 180 ml of 70% ethanol to the beaker containing 20g of pulverized peels. This step marks the beginning of the maceration. The ethanolic solution was then magnetically stirred. A magnetic stirrer ensured the ethanolic solvent was even when mixing with the sample material. The magnetic stirrer helps maintain a constant interaction between the ethanol and the sample through continuous stirring of the mixture. This process can enhance the target compounds' solubilization and increase the extraction process's overall yield. The solution underwent magnetic stirring for 5 minutes at 90c. The solution was left to soak for 24 hours for the flavonoids to yield accordingly, wherein the substance was stored in a dark and dry area to ensure the quality of the solution.

After 24 hours of soaking, a stirring rod was utilized in the experiment to mix the solution to avoid clumping of sediments. Then, the researchers transferred the solution to multiple test tubes in preparation for the ethanolic water bath extraction. The solution's test tubes underwent water bath treatment for 30 minutes at 90c. After ethanolic water bath treatment, the viscous extract remained at the bottom of the test tubes. This viscous extract contains the targeted compounds that will exhibit anti-angiogenic activity. After ethanolic-water bath extraction, the test tubes were centrifuged for 3 minutes at 30 rpm to ensure that the viscous extract would be sedimented and isolated. The researchers then transferred viscous extract into a 1000ml beaker to prepare the stock solution. Implementing a 1:10 ratio, 20g of the extract was diluted with 180ml of distilled water to derive the *Linum flavum* (Golden Flaxseed) stock solution. The next step included pouring the stock solution into smaller-sized 200ml beakers to prepare the respective dilutions.

Then, the researchers prepared a series of ethanolic extract concentrations varying from 10%, 30%, 50%, and 70%. Both plants undergo ethanolic extraction to have equal footing using a 70% ethanolic extraction. (Barluado et al., 2013), Ethanol extract for Citrus macrocarpa peels uses a ratio of 1:10, and per 0.10 g/ml of extract, 70% ethanol was used in the study to macerate the seeds. Thus, the study also implemented a 1:10 solvent ratio for *Linum flavum* (Golden Flaxseed).

The researchers utilized 20g of *Linum flavum* (Golden Flaxseed). Viscous extract and 180ml of 70% ethanol as a solvent. Thus, the researchers yielded a total of 200 plant extracts. The stock solution was further diluted into four different concentrations to contextualize the concentration to the study's objectives. Below is the computation for each concentration:

Let,
V_s = Volume of stock solution
V_w = Volume of distilled water
V_t = Total Volume after dilution
v/v% = Volume Percent

The formula for the total Volume (V_t) can be expressed as the sum of the Volume of the stock solution (V_s) and the Volume of distilled water (V_w):

$V_t = V_s + V_w$

Table 3. Computation of dilution of the stock solution *Linum flavum*

v/v%	Vs+Vw	Vt
For a 10% concentration	$10\%=\left(\frac{10}{100}\right)$ 200ml Vs + 180mL of distilled water	200mL of 10% diluted stock solution
For a 30% concentration	$30\%=\left(\frac{30}{100}\right)$ 200ml Vs + 140mL of distilled water	200mL of 30% diluted stock solution
For a 50% concentration	$50\%=\left(\frac{50}{100}\right)$ 200ml Vs + 100mL of distilled water	200mL of 50% diluted stock solution
For a 70% concentration	$70\%=\left(\frac{70}{100}\right)$ 200ml Vs + 60mL of distilled water	200mL of 70% diluted stock solution

Table 4. Dilution of the stock solution *Linum flavum*

v/v%	Stock Solution	Distilled Water (Diluent)
10% concentration	20ml	180ml
30% concentration	60ml	140ml
50% concentration	100ml	100ml
70% concentration	140ml	60ml

As shown in Table 3 and Table 4, to dilute the stock solution to a 10% concentration, a portion equal to one-tenth of the original stock solution is combined with approximately 180 mL of distilled water. This mixture yields a final volume of 200 ml, achieving the desired 10% concentration. The dilution process is straightforward, adhering to the formula derived from the mass conservation principle.

Similarly, when aiming for a 30% concentration, three-tenths of the stock solution was diluted with 170mL of distilled water. The result is a 200 ml final volume of a 30% diluted stock solution. The researchers applied the same approach for concentrations of 50% and 70%, mixing five-tenths of the stock solution with approximately 100mL of distilled water and seven-tenths of the stock solution mixed with approximately 60mL of distilled water to maintain the final Volume at 200ml for each case. After dilution, the researchers used an amber bottle to store the total solution. This process demonstrates the consistency and adaptability of the dilution formula.

The researchers assessed the anti-angiogenic activity of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) through Duck Embryo Chorioallantoic Membrane (CAM) Assay, wherein the researchers will adapt the procedure utilized by (Barluado et al., 2013) in the assessment of the anti-angiogenesis activity of *Citrus microcarpa* and a study conducted by (Anglo-Ojeda, 2022) in the comparative measures of the anti-angiogenic activity of ascorbic acid and Vitamin B complex. After the acquisition and incubation of the fertilized duck from a licensed fertilized duck egg raiser from Pateros, Metro Manila, the researchers prepared it for experimentation.

The study used a flashlight to assess air sac size in each egg. Carefully avoiding damage to the eggs, the researchers used a small drill to create a small window on the shell to expose the CAM. The concentrations start from 10% and increase in increments of 20% (i.e., 10%, 30%, 50%, and 70%). The researchers used three fertilized duck embryos for each concentration level as experimental units. As the concentration increased, they added three fertilized duck embryos for every increment of 20%. In one run, the researchers used 30 eggs per plant, then one for the positive control and one for the negative control for each concentration. Hence, the researchers used 76 eggs per 1 experimental run. The researchers were conducting two experimental trials. Therefore, the study utilized a total of 228 eggs. The researchers added 0.1mL of each ethanolic extract concentration to their respective numbers of fertilized duck eggs. The study also included controls, wherein standard saline solution is the negative control, and ascorbic acid is the positive control. After the application, the egg window was closed using a paraffin film and then incubated for approximately 72 hours to allow time for angiogenic responses. After incubating, carefully crack the egg and place it in a sterile petri dish. Then, interpret the results. Determining the minimum concentration includes the minimum ethanolic extract concentration that can inhibit angiogenesis, while the potent concentration exhibits the most effective anti-angiogenic activity.

Since the researchers utilized numerous materials in this study, it is essential to consider that there are varying methods to dispose of these wastes. Organic wastes, such as eggshells, unused duck embryos, and plant residues, should be disposed of in biohazard bins or composting facilities. Moreover, ethanol residues should be stored based on the instructions included in the reagent. Other wastes, such as Personal Protective Equipment and contaminated materials, should be disposed of in the biohazard bin. The researchers disposed of all the sharps materials utilized in the study in puncture-resistant containers labeled for sharps disposal. The researchers stored all unused reagents or samples according to their specific handling instructions.

After the corresponding incubation period of the fertilized duck eggs, they removed the eggshells to assess the CAM fully; then, they placed the embryos in a sterile petri dish. The researchers took an image of each duck embryo using a phone camera. Then, they uploaded the captured images to the ImageJ Software to manually count the blood vessels and generate results for the mean values of the vessels. Then, the researchers will assess various angiogenic parameters, including blood vessel density, branching, and the overall vascular network. The researchers evaluated the overall angiogenic parameters using the Image J Software, which scientists widely use to allow users to visualize, inspect, quantify, and validate scientific image data. The software can track and visualize the biological entities to interpret the imaging data. (Schroeder, et al., 2021). The results may then be analyzed and interpreted using various statistical tools. A decrease in blood vessel formation may indicate the plant's anti-angiogenic activity.

2.5 Ethical Considerations

In conducting a Comparative Study on the Anti-Angiogenesis Activity of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) Extracts using the Duck Embryo Chorioallantoic Membrane (CAM) Assay, the primary importance is ensuring the safety of all individuals involved, particularly students. Strict measures ensure the safe handling of samples and living organisms based on established protocols and institutional guidelines. Students received comprehensive guidance on laboratory safety practices, encompassing proper handling techniques, utilizing personal protective equipment (PPE), and strict adherence to established sample handling and disposal protocols. Supervision and monitoring procedures are regularly in place to evaluate and handle any safety or ethical issues that could surface throughout the study. Students prioritizing safety and moral issues are better equipped to perform their research ethically.

3.0 Results and Discussion

3.1 Anti-angiogenic Activity of *Citrus aurantium* Concentrations

The concentrations of *Citrus aurantium* peels that exhibit anti-angiogenic activity are assessed by the number of new blood vessels formed. Table 5 shows the four concentrations of *Citrus aurantium* and the positive control. It shows the mean of the four concentrations and the positive control, which is 84.0789. The data allowed the comparison of the mean of the positive control against the four concentrations.

Table 5. Descriptive statistics of the anti-angiogenic activity of *Citrus aurantium* concentrations

10%	30%	50%	70%	General Weighted Mean
89.64	91.21	89.74	123.42	**98.50**

The 10% concentration yielded a mean of 89.64. The 30% concentration yielded a mean of 91.21. The 50% yielded a mean of 89.74. Lastly, 70% concentration yielded a mean of 123.42. In the context of this study, the results of the concentrations should be similar to the positive control for it to be reproducible. A closer mean would imply that the results of each concentration are consistently identical to the positive control, with a margin error of +/-5. Similarly, substantial results of anti-angiogenesis activity range from 46.55+_ to 89.05 +_ 0.4343% at concentrations of 10% to 50% (Barluado et al., 2013). Comparatively, the results of the experiment exhibited 89.64 at 10%. This correlates to the findings of Barluado et al. (2013). This implies that substantial results have already been achieved, at 10%, for this group.

3.2 Anti-angiogenic Activity of *Linum flavum* Concentrations

Table 6 shows the four concentrations of *Linum flavum* (golden flaxseed) and the positive control. It shows the mean of the four concentrations and the positive control, which is 84.0789. The data allowed comparing the mean of the positive control against the four concentrations.

Table 6. Descriptive statistics of the anti-angiogenic activity of *Linum flavum* concentrations

10%	30%	50%	70%	General Weighted Mean
109.23	87.32	119.38	134.63	**112.64**

The 10% concentration yielded a mean of 109.23. The 30% concentration yielded a mean of 87.32. The 50% yielded a mean of 119.38. Lastly, 70% concentration yielded a mean of 134.63. In the context of this study, the results of the concentrations should be similar to the positive control for it to be reproducible. A closer mean would imply that the results of each concentration are consistently identical to the positive control, with a margin error of +/- 5. Similarly, substantial results of anti-angiogenesis activity range from 46.55+_ to 89.05 +_ 0.4343% at concentrations of 10% to 50% (Barluado et al., 2013). Comparatively, the results of the experiment exhibited 87.32 at 30%. This correlates to the findings of Barluado et al. (2013). This implies that substantial results have already been achieved, at 30%, for this group.

3.3 Anti-angiogenic Activity of *Citrus aurantium* Concentrations (Positive Control)

Table 7 shows the corresponding concentrations and their correlation to the positive control. In the descriptive statistics table, the first column indicates that only 10% of the solution of *Citrus aurantium* (Dalandan) shows a closer value to the mean of the positive control. This value indicates that a 10% concentration of *Citrus aurantium* (Dalandan) exhibits the most potent anti-angiogenic activity in comparison to the mean of the positive control. Similarly, substantial results of anti-angiogenesis activity range from 46.55+_ to 89.05 +_ 0.4343% at concentrations of 10% to 50% (Barluado et al., 2013). Comparatively, the results of the experiment exhibited 89.64 at 10%.

Table 7. Descriptive statistics of the anti-angiogenic activity of *Citrus aurantium* concentrations (positive control)

10%	30%	50%	70%	Mean of the Positive Control
89.64	91.21	89.74	123.42	**84.079**

This correlates to the findings of Barluado et al. (2013). This implies that substantial results have already been achieved, at 30%, for this group. The data indicates that a 10% concentration of *Citrus aurantium* (Dalandan) peel extract exhibits the most potent anti-angiogenic activity, closely aligning with the mean of the positive control. According to Barluado et al. (2013), significant anti-angiogenesis activity, ranging from 46.55% to 89.05%, was observed between 10% and 50% concentrations, supporting the finding that lower concentrations, particularly 10%, demonstrate more substantial anti-angiogenic effects. Thus, the 10% concentration is the most effective compared to the other concentrations tested.

3.4 Anti-angiogenic Activity of *Linum flavum* Concentrations (Positive Control)

Table 8 shows the corresponding concentrations and their correlation to the positive control. In the descriptive statistics table, the first column indicates that only 30% of the solution of *Linum flavum* (Golden Flaxseed) shows a closer value to the mean of the positive control. This value indicates that a 30% concentration of *Linum flavum* (Golden Flaxseed) exhibits the most potent anti-angiogenic activity in comparison to the mean of the positive control. Similarly, substantial results of anti-angiogenesis activity range from 46.55+_ to 89.05 +_ 0.4343% at concentrations of 10% to 50% (Barluado et al., 2013).

Table 8. Descriptive statistics of the anti-angiogenic activity of *Linum flavum* concentrations (positive control)

10%	30%	50%	70%	Mean of the Positive Control
109.23	87.32	119.38	134.63	**84.079**

Comparatively, the results of the experiment exhibited 87.32 at 30%. This correlates to the findings of Barluado et al. (2013). This implies that substantial results have already been achieved, at 30%, for this group. The data indicates that a 30% concentration of *Linum flavum* (Golden Flaxseed) exhibits the most potent anti-angiogenic activity, with a mean value of 87.32, closely aligning with the mean of the positive control (84.079). This suggests that 30% is the optimal concentration for inhibiting angiogenesis compared to other concentrations tested. The concentrations of 10%, 50%, and 70% are higher values (109.23, 119.38, and 134.63, respectively), indicating less effectiveness than the positive control. According to Barluado et al. (2013), significant anti-angiogenesis activity ranges from 46.55% to 89.05% at concentrations between 10% and 50%, which supports the finding that 30%

concentration in this experiment is the most effective. Therefore, substantial anti-angiogenic results are achieved at 30%, which aligns with previous findings.

3.5 Comparison Between the Anti-angiogenic Activity of *Citrus aurantium* and *Linum flavum*

Table 9 compares the anti-angiogenic activity of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) ethanolic extracts based on multiple regression analysis using the Chorioallantoic Membrane (CAM) assay.

Table 9. Multiple regression analysis for the anti-angiogenic activity of *Citrus aurantium* and *Linum flavum*

***Citrus aurantium* (Dalandan)**		***Linum flavum* (Golden Flaxseed)**	
Multiple R	0.1506413	Multiple R	0.70349206
R Square	0.0226928	R Square	0.494901078
Adjusted R Square	-0.46596	Adjusted R Square	0.242351617
Standard Error	20.13165	Standard Error	17.27548247
Observation	4	Observation	4

The study compares the anti-angiogenic activity of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) ethanolic extracts using the Chorioallantoic Membrane (CAM) assay. The regression analysis results indicate that *Linum flavum* shows a more substantial anti-angiogenic effect, with a Multiple R-value of 0.7035, suggesting a moderate to strong correlation between its concentration and anti-angiogenic activity. Moreover, the model for *Linum flavum* explains 49.49% of the variation in activity ($R^2 = 0.4949$), making it a more effective agent in inhibiting blood vessel formation under the assay conditions. In contrast, *Citrus aurantium* demonstrated a much weaker correlation (Multiple R = 0.1506), with its model explaining only 2.27% of the activity variation ($R^2 = 0.0227$), indicating that it may be less effective in inhibiting angiogenesis in this setup.

The higher adjusted R^2 for *Linum flavum* (0.2424) compared to the negative value for *Citrus aurantium* suggests that the former has some predictive power, while the latter does not. These results point to *Linum flavum* as the more promising anti-angiogenic agent of the two, though the high standard error for both models suggests that additional research is necessary to increase the reliability of these findings. Expanding the number of observations and refining experimental conditions, such as varying concentrations or using different extraction methods, could further validate these results and clarify the potential anti-angiogenic benefits of these plant extracts. Also, the table shows that C. aurantium yielded an R-value of 0.022, indicating a significant difference in the positive control since the value is <0.05. On the other hand, L. flavum yielded an F value of 0.494, indicating no significant difference in the positive control since the value is >0.05.

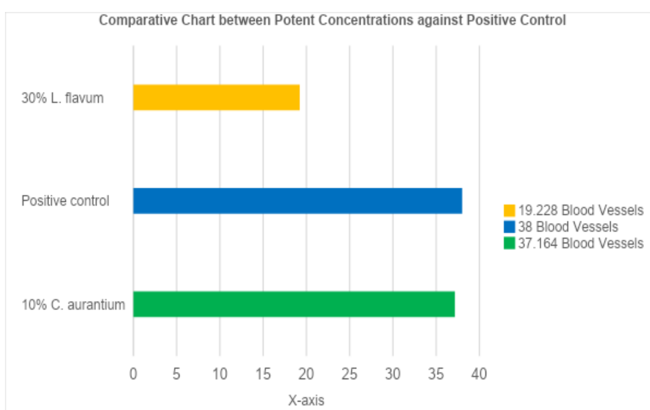

Figure 1. The comparative chart between potent concentrations against positive control

Figure 1 compares the results of the most potent concentrations with the positive control. Multiple regression accounts for the variation of the results against the positive control. Data shows that the positive control contains 38 blood vessels. The positive control was the benchmark the researchers tested with the corresponding concentrations. 30% *L. flavum* yields an R-squared value of 0.494 against the positive control. Meanwhile, 10 % *C. aurantium* yields an R squared value of 0.022 variation against the positive control. Therefore, it means that *C. aurantium* is closer to the benchmark than *L. flavum.* In the context of this research, a value of <0.05 in the R squared value is a much better figure as it is closer to the benchmark. If it has a lower value, it has a lesser difference towards the positive control. Having said this, a lower R squared value means that it is more predictable and, therefore, more consistent in cases of reproducing results, as a higher variation would mean it would yield different outcomes.

Citrus aurantium (Dalandan) and *Linum flavum* (Golden Flaxseed) exhibit anti-angiogenic activity. Among the four concentrations of *Citrus aurantium* (Dalandan), the 10% concentration, which has a mean value of 89.64, has the closest relationship to the mean value of positive control, 84.0789. On the other hand, among the four concentrations of *Linum flavum,* the 30% concentration, which has a mean value of 87.32, has the closest relationship to the mean value of positive control, 84.079. In Barluado and Angloojeda's study, the anti-angiogenic activity measured the extract's ability to inhibit the growth of branches of blood vessels in duck embryo CAM assay. The 30% concentration of the plant extract showed the least number of blood vessel formation.

Regarding the most potent activity, the Sig column indicates that the 10% solution of *Citrus aurantium* (Dalandan) independent variable coefficients is statistically significant with a mean value of 89.64. This value indicates a significant difference between the 10% ethanolic extract of *Citrus aurantium* (Dalandan) and the positive control. On the other hand, *Linum flavum* (Golden Flaxseed), the Sig column indicates that the 30% solutions of *Linum flavum* (Golden Flaxseed) are statistically significant with a mean value of 87.32. This value indicates a significant difference between 30% ethanolic extracts of *Linum flavum* (Golden Flaxseed) and the positive control.

These values correlate with the anti-angiogenic activity of the most potent concentration of *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed). The concentration with a mean closest to the positive control is 10%, consistent with the data indicated by the descriptive statistics, which shows a significant difference compared to the positive control. On the other hand, the most potent anti-angiogenic activity of *Linum flavum* (Golden Flaxseed) is the 30% concentration, also consistent with the result in the descriptive statistics, which shows a significant difference compared to the positive control.

Moreover, the researchers assessed the overall effectiveness of *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) through the R squared value of the respective concentrations' mean value relative to the positive control's mean. A value of 0.022 indicated the relationship between the effectiveness of *Citrus aurantium* (Dalandan) in comparison to the mean of the positive control. On the other hand, a value of 0.494 indicated the variation in the effectiveness of *Linum flavum* (Golden Flaxseed) in comparison to the mean of the positive control. Overall, in the context of the study, the positive control determines how the blood vessels should appear after treatment. The data quantifies that 10 % *C. aurantium* has 37.164 observable blood vessels, while 30% *L. flavum* has 19.228 observable blood vessels.

Furthermore, it is evident that *Citrus aurantium* (Dalandan) at a 10% concentration exhibited vigorous anti-angiogenic activity, closely resembling the control, thus indicating more consistent and dependable outcomes compared to *Linum flavum* (Golden Flaxseed) at 30%, which displayed greater variability. The lower R-squared value for *Citrus aurantium* (0.022) indicates a closer alignment with the control. This observation is supported by the study of Barluado et al. (2013), which showed that *Citrus aurantium* is most effective at a concentration of 10%, while *Linum flavum* requires higher concentrations. Both have anti-angiogenic effects, but *Citrus aurantium* is more effective at lower concentrations.

Table 10. Ethanolic extractions and number of embryo deaths

Citrus aurantium	Embryo Death	*Linum flavum*	Embryo Death
10%	0	10%	2
30%	0	30%	0
50%	1	50%	6
70%	3	70%	6

Additionally, Table 10 shows the embryonic death of observed Duck Eggs in the in vitro CAM Assay. The data shows a proportional death increase for each increasing concentration. However, this is not the case for *Linum flavum (Golden Flaxseed), as there were no deaths from the* 30% concentration. The first row shows both data for the 10% concentration of each plant. *Citrus aurantium* (Dalandan) exhibits no embryonic death. Meanwhile, the *Linum* *flavum* (Golden Flaxseed) exhibits two embryonic deaths. As observed in the second row, both plants had no embryonic deaths in the 30% concentration. For 50% concentration, *Citrus aurantium* (Dalandan) exhibited one embryonic death. On the other hand, six embryonic deaths were observable in vitro for *Linum flavum* (Golden Flaxseed). Lastly, the fourth row shows 70% concentrations for each plant. *Citrus aurantium* (Dalandan) exhibited three embryonic deaths. Meanwhile, *Linum flavum* (Golden Flaxseed) exhibited six embryonic deaths in vitro.

As opposed to the study conducted by Barluado et al. in the anti-angiogenic activity of *Citrus macrocarpa, in 10% concentration, there are no deaths exhibited, which is also similar to* the embryonic deaths for 10% concentration in *Citrus aurantium* (Dalandan), which is 0. On the other hand, 10% ethanolic extraction of *Linum flavum* (Golden Flaxseed) showed two embryonic deaths. For the 30% concentration, *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) showed no deaths, compared to the embryonic deaths in the anti-angiogenic activity of *Cirus macrocarpa*, which is 3. Next, for the 50% concentration, *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) showed 1 and 6 embryonic deaths, respectively, as opposed to the six deaths of anti-angiogenic activity of *Cirus macrocarpa.* Lastly, for the 70% concentration, *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) showed 3 and 6 embryonic deaths, as opposed to the 70% concentration of *Citrus macrocarpa*, which exhibited 12 deaths.

Also, the table demonstrates that *Citrus aurantium* (Dalandan) did not cause any embryo deaths at a 10% concentration, while *Linum flavum* (Golden Flaxseed) resulted in two deaths at this concentration. At higher concentrations (50% and 70%), both extracts led to more deaths, with *Linum flavum* causing significantly more. This indicates that *Citrus aurantium* is safer at lower concentrations, while *Linum flavum* may become more toxic as concentrations increase. These findings are consistent with studies such as Barluado et al. (2013), which show that plant extracts can exhibit cytotoxic effects at higher doses, emphasizing the importance of controlling concentrations in therapeutic applications.

4.0 Conclusion

The results of the experiment acquired by the researchers from conducting the CAM Assay on fertilized duck eggs utilizing ethanolic extractions of *Citrus aurantium* (Dalandan) peels and *Linum flavum* (Golden Flaxseed) showed that both plants exhibit anti-angiogenic activity. The most potent concentrations were 10% for *Citrus aurantium* (Dalandan) peels and 30% for *Linum flavum* (Golden Flaxseed). The researchers concluded that *Citrus aurantium* (Dalandan) and *Linum flavum* (Golden Flaxseed) exhibit anti-angiogenic activity at specific concentrations. Specifically, the most potent concentration for *Citrus aurantium* (Dalandan) is 10%, while for *Linum flavum* (Golden Flaxseed), it is 30%. Overall, *Citrus aurantium* (Dalandan) exhibited the most effective anti-angiogenic activity compared to *Linum flavum* (Golden Flaxseed). Based on this study, both plants can be a good source of anti-cancer therapy, but further research is needed to establish the best dosage for anti-angiogenesis activity.

5.0 Contribution of Authors

Raymund J. Capagas, RMT, MPH — supervising, conceptualizing, finalizing; Mary Jobhel Calleung — editing and writing; Daschel Alliyah Diega — editing, writing, and data analysis; Aaliyah Dominique Gonzales — editing and writing; Benedict Isleta — editing, writing, and data analysis, and Dhana San Agustin — editing and writing.

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7.0 Conflict of Interests

The authors declare no conflict of interest.

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