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作品名稱 **Phytochemical screening and evaluation of antiangiogenic properties of sapinit (Rubus fraxinifolius) fruit crude extracts**

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作者照片

ABSTRACT

Plants are potential low-cost alternatives for cancer treatment. *Rubus fraxinifolius* or "sapinit" has been found to possess phytochemicals with anti-cancer potential. This project aimed to evaluate the antiangiogenic properties of methanolic *R. fraxinifolius* fruit crude extracts through the chick chorioallantoic membrane (CAM) assay. Through phytochemical screening, leucoanthocyanins, phenols, and tannins were detected. For the CAM assay, 10, 20, and 30 μg/μL extracts, distilled water, methanol, and retinoic acid were applied on 60 ten-day-old chicken eggs. The CAM photographs were analyzed using ImageJ Software. The mean percent inhibitions (MPI) of total length and vascular density from both analyses were subjected to One-Way Analysis of Variance (ANOVA). The ANOVA for the MPI of total length, followed by a Tukey post hoc test, show that only retinoic acid treatment has significantly higher MPI ($p = 0.0010$). Meanwhile, the results for the MPI of vascular density show no significant differences between all groups ($p = 0.1630$). It is possible that the concentrations used in the study may not be the concentrations needed to achieve optimal antiangiogenesis. The results may also be due to the absence of phytochemicals that exhibit significant antiangiogenic properties such as alkaloids. Lower concentrations and isolated phytochemicals may also be tested.

INTRODUCTION

Background of the Study

Cancer is a noncommunicable disease that remains to be one of the leading causes of death not only in the Philippines (Department of Health, n.d.) but even in the world. According to the World Health Organization (2018) or WHO, cancer caused one out of six deaths in the world. About 9.6 million people died of cancer in 2018, and 70% of these deaths are in low- and middle-income countries (WHO, 2018). The leading causes of cancer death in 2018 were cancers of the lung, breast, prostate, colon, stomach, and liver (Bray et al., 2018).

In the Philippines, the International Agency for Research on Cancer (2018) or IARC recorded over 140,000 new cases of cancer in 2018. This organization also recorded nearly 87,000 deaths caused by cancer in the same year out of nearly 590,000 deaths (Philippine Statistics Authority, 2019), with the leading causes being cancers of the breast, lung, colon, and liver (IARC, 2018).

Despite the number of deaths that cancer causes in the world annually, cancer treatment remains inaccessible due to its costliness. Radiotherapy and chemotherapy sessions are given over a long period of time, and follow-up care is necessary to ensure effective cancer treatment. According to a study conducted by Ngelangel et al. (2018) regarding the economic effects of cancer on Filipino patients, 40.6% of patients faced financial problems due to cancer treatment. Cancer development, specifically the transportation of oxygen and nutrients necessary for tumor growth, relies on the formation of blood vessels from pre-existing blood vessels or angiogenesis (Camposano, Dela Torre, Laxamana, & Larcia, 2016). Commercially available synthetic treatments for the inhibition of blood vessel growth, or antiangiogenesis, are also expensive and ineffective in some cases (Camposano et al., 2016).

Eating fruits and vegetables that are rich in bioactive compounds can reduce the effects of cancer, as well as other chronic diseases (Hjartåker, Knudsen, Tretli, & Weiderpass, 2014). Among these healthy fruits are berries, which are particularly rich in phytochemicals (Jimenez-Garcia et. al, 2018). Studies of *Rubus* fruits have shown the presence of phytochemicals and antioxidant and anticancer activities. Different dosages of methanolic extracts of *Rubus* spp. significantly affected the viability of Caco-2 cell lines (Muniyandi et al., 2019).

Rubus fraxinifolius, more commonly known as "sapinit" or the Philippine Wild Raspberry, is a shrub with bright red-orange fruits that can mostly be found in Mt. Banahaw in Quezon and in Laguna. It was discovered by Dionisio Pullan, an overseas Filipino worker in Australia. In a phytochemical analysis conducted by the Industrial Technology Development Institute and the University of the Philippines Los Banos-Biotech funded by the Bureau of Agricultural Research (BAR) program, it was discovered that the "sapinit is rich in anticancer phytochemicals including leucoanthocyanins, anthraquinones, saponins, deoxysugars, free fatty acids, hydrolysable tannins (inhibitors of HIV duplication), unsaturated steroids, and benzopyrone nucleus." ("Sapinit: Philippine Wild Raspberry," 2018, para. 2). While a study by Alvarez, Caunca, & Nolido (2013) tested the antimicrobial properties of *R. rosifolius* sapinit fruit extracts, there have been limited studies on the anticancer properties of *R. fraxinifolius* extracts, one of which is antiangiogenesis.

Objectives of the Study

This study aimed to evaluate the antiangiogenic properties of sapinit (*R. fraxinifolius*) fruit crude extract using the chick chorioallantoic membrane (CAM) assay to identify its potential anti-cancer properties. The presence of antiangiogenic properties may suggest that sapinit extract is a possible, less costly alternative to costly anti-cancer treatments.

This study also aimed to conduct a qualitative phytochemical screening of sapinit fruits to detect the following secondary metabolites: alkaloids, anthraquinones, flavonoids, leucoanthocyanins, phenols, sterols, and tannins, and to to compare the bioactivities of different methanolic extract concentrations.

Significance of the Study

Evaluating the antiangiogenic properties of sapinit will contribute to the complete profiling of the bioactivities of the fruit. Further investigation on the health benefits of sapinit may aid in drug development and the development of other health-related products. A less costly source of cancer treatment would benefit cancer patients the most.

Aside from contributing to the medical industry, presenting the different benefits of sapinit will also lead to better and more widespread cultivation of the fruit. Being an environmentally sustainable plant, it will be able to provide livelihoods to Filipino communities.

Scope and Limitations

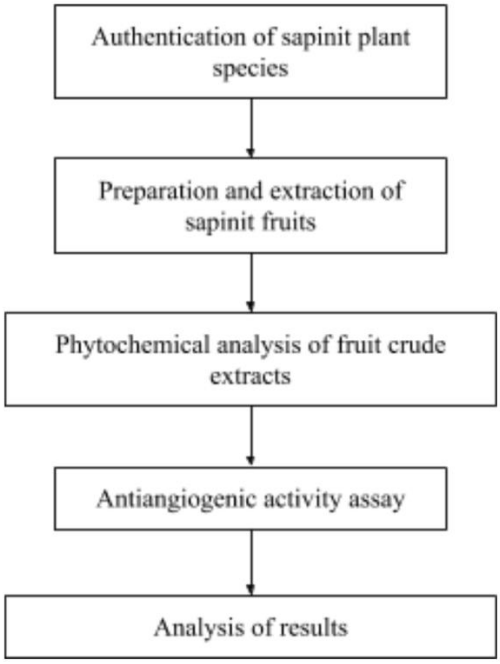
The study focused on evaluating the antiangiogenic properties of *R. fraxinifolius* fruit crude extracts using the CAM assay. Phytochemical analysis of the fruit crude extract was also conducted using qualitative confirmatory tests to detect the presence of certain phytochemicals.

Although the presence of different phytochemicals in the fruit crude extract were determined through qualitative analysis, the researchers were not able to isolate the phytochemicals due to time constraints. Since *R. fraxinifolius* is a seasonal fruit, procurement and extraction of the fruit can only be done in January until March. Although it will not be covered in this project, fractionation of the extract is recommended to isolate specific phytochemicals.

Antiangiogenesis assays, as well as other tests, may be performed on these phytochemicals once they are isolated. Due to limitations on laboratory activities during the COVID-19 pandemic, the CAM assay could not be repeated to validate the results.

METHODOLOGY

Process Flowchart



Authentication of sapinit plant species

Procurement of sapinit plant part

A sample of a stem with a leaf from a *Rubus fraxinifolius* (sapinit) plant was procured from Bangkong Kahoy Valley Nature Retreat and Field Study Center in Dolores, Quezon.

Identification of sapinit species

The sample of sapinit plant procured was brought to and authenticated by the University of the Philippines-Diliman (UPD) Institute of Biology Herbarium in Quezon City.

Preparation and extraction of sapinit fruits

Procurement of sapinit fruits

Two (2) kilograms of sapinit fruit harvested on the same date (January 26, 2020) were procured from Bangkong Kahoy Valley after authentication.

Preparation of powdered sapinit fruits

The sapinit fruit was oven-dried for 72 hours at 40ºC. Afterwards, it was left to cool and crushed into smaller pieces. The mashed fruits were then divided into three 500 mL beakers, and the beakers were submerged in a hot water bath at 40ºC for two hours to ensure evaporation of the water content.

Maceration and rotary vacuum evaporation of powdered sapinit fruits

A 0.01% (v/v) hydrochloric acid (HCl) in methanol solution was prepared by adding 0.45 mL HCl to 450 mL methanol (Rodriguez-Saona & Wrolstad, 2001). The beakers containing the fruit samples were each submerged in 150 mL acidified methanol. The mixtures were left to macerate overnight then filtered using filter paper and a glass funnel. The filtrate was placed in a flask, and the methanol was evaporated using a rotary evaporator at 40ºC. After rotary evaporation, the extract was stored in vials. The maceration process was repeated, and the plant material and solvent used in the first run of maceration were reused. The mixture was left overnight again, and the filtration and evaporation process were repeated. The final extract was stored in vials, and the leftover solvent and plant material were discarded.

Lyophilization of leftover fruit crude extract

The fruit crude extract was brought to De La Salle University Manila for lyophilization.

Phytochemical analysis of fruit crude extracts

Dragendorff's test for alkaloids

Concentrated hydrochloric acid with a volume of 0.85 mL was mixed with 4.15 mL of distilled water (Aguinaldo, Espeso, Guevara, & Nonato, 2004). One (1) g of sapinit extract was stirred into the diluted hydrochloric acid while heating for five (5) minutes. After cooling, 0.5 g of sodium chloride was added to the solution. The solution was filtered, and one (1) mL of the filtrate was mixed with 2-3 drops of Dragendorff's reagent (Aguinaldo et al., 2004). The mixture was then observed to see if an orange color change indicating the presence of alkaloids occurred (Aguinaldo et al., 2004).

Ferric chloride test for tannins

One (1) g sapinit extract was mixed with 20 mL of hot distilled water (Aguinaldo et al., 2004). One (1) g of sodium chloride was then dissolved in nine (9) mL of distilled water, and five (5) drops of this mixture were added to the diluted extract. The solution was filtered (Aguinaldo et al., 2004). 0.1g of ferric chloride was dissolved in ten (10) mL of distilled water. 0.75 mL of ferric chloride solution was dissolved in 25 mL distilled water. Three (3) drops of the ferric chloride reagent were added to the extract (Aguinaldo et al., 2004). The mixture was then observed to see if a blue-black color change indicating the presence of hydrolyzable tannins or a brownish-green color change indicating the presence of condensed tannins occurred (Aguinaldo et al., 2004).

Ellagic acid test for phenols

Sodium nitrite with a volume of 0.5 mL was added to ten (10) mL of distilled water (Sheel, Nisha, & Kumar, 2014). Glacial acetic acid with a volume of 0.5 mL was then added to ten (10) mL of distilled water. A small amount of sapinit extract was added into a test tube. A few drops of 5% (w/v) glacial acetic acid and 5% (w/v) sodium nitrite solution were added to the extract (Sheel

et al., 2014). The mixture was then observed to see if a muddy brown color change indicating the presence of phenols occurred (Sheel et al., 2014).

Borntrager's reaction for free anthraquinones

Two (2) mL of sapinit extract and five (5) mL chloroform were added to a dry test tube (Le, 2019). An equal volume of 10% ammonia solution was added to the test tube, and the mixture was shaken. The mixture was then observed to see if a pink, violet, or red layer indicating the presence of free anthraquinones appeared (Le, 2019).

Salkowski test for sterols

Two (2) mL of sapinit extract was added to a test tube (Sheel et al., 2014). Two (2) mL of chloroform and two (2) mL of concentrated sulfuric acid were then added, and the mixture was shaken. The mixture was then observed to see if a red chloroform layer and greenish yellow fluorescent acid layer indicating the presence of sterols appeared (Sheel et al., 2014).

Ammonium and aluminum chloride test for flavonoids

A small amount of sapinit extract was mixed with ten (10) mL of ethyl acetate in a beaker. The beaker was heated in boiling water for three (3) minutes. After heating, the mixture was filtered and divided into two test tubes (Sheel et al., 2014).

One (1) mL of 1% dilute ammonia solution was added to the first test tube, and the mixture was shaken. The layers were left to separate, and the mixture was observed to see if a yellow ammonia layer indicating the presence of flavonoids appeared (Sheel et al., 2014).

One (1) mL of 1% aluminum chloride solution was also added to the second test tube, and the mixture was shaken. The mixture was then observed to see if a light yellow color change indicating the presence of flavonoids appeared (Sheel et al., 2014).

Test for leucoanthocyanins

One (1) mL of sapinit extract was mixed with one (1) mL of isoamyl alcohol (Bansode & Salalkar, 2015) in three (3) test tubes. The mixture was then observed to see if a red upper layer indicating the presence of leucoanthocyanins appeared (Bansode & Salalkar, 2015).

Antiangiogenic activity assay

Preparation of dilutions of the extracts

Three treatments, ten (10) mL each of the 10, 20, and 30 µg/µL concentrations of the extracts, were prepared. For the 10 µg/µL concentration, 100 mg of sapinit extract was mixed with 10 mL of distilled water. For the 20 µg/µL concentration, 200 mg of sapinit extract was mixed with 10 mL of distilled water. For the 30 µg/µL concentration, 300 mg of sapinit extract was mixed with 10 mL of distilled water.

Procurement and preparation of chicken eggs

Sixty (60) 5-day old chicken eggs were procured from Jing-Jing's Balut located along Epifanio de los Santos Avenue, Balintawak, Quezon City.

Chick chorioallantoic membrane (CAM) assay

The 60 5-day old fertilized chicken eggs were cleaned with 70% ethanol and kept in an incubator at 37.8°C with a humidity of 70% for five (5) days (Tantiado & Tan, 2012). The eggs were brought out from the incubator, and egg candling was performed using a phone flashlight for each 10-day old egg. A 1 cm x 1 cm window was made on each egg using a scalpel (Luay, Gonzaga, Po, & Arollado, 2018). Photos of the CAM of the eggs were taken (Luay et al., 2018). Ten (10) µL of each treatment was applied dropwise using a capillary pipette to 10 filter paper discs each. The six (6) treatments were 10, 20, and 30 µg/µL concentrations of the extract, distilled water, methanol, and retinoic acid. A filter paper disc was placed directly over the CAM through the

window using forceps. The windows were covered using parafilm and the eggs were incubated (Luay et al., 2018). After two (2) days, the eggs were removed from the incubator. The parafilms were removed and photos of the CAM of all eggs were taken. The photos were analyzed using ImageJ Software (Luay et al., 2018).

Computation of % inhibition of blood vessels

Using the data in ImageJ, the percent growth of the blood vessels in terms of total length and vascular density were computed. Given the total length or vascular density of the untreated CAM (U) and the total length or vascular density of the treated CAM (T), the percent growth of blood vessels in the the CAM treated by each of the six treatments was computed using the formula (Luay et al., 2018):

$$\% \text{ growth} = [(T - U) / T] \times 100\% \qquad \text{(Eq. 2)}$$

Given the percent growth of blood vessels in the CAM treated by distilled water (W) and the percent growth of blood vessels in the CAM treated by one of the five other treatments (T), the percent inhibition were computed using the formula with positive mean percent inhibition indicating a decrease in blood vessel growth or antiangiogenesis (Luay et al., 2018):

$$\% \text{ inhibition} = [(W - T) / W] \times 100\% \qquad \text{(Eq. 3)}$$

Analysis of results

Compilation and presentation of results from the phytochemical screening

The results from the phytochemical screening were compiled and presented in a table.

Statistical analysis of the data from the CAM Assay

Statistical analysis was conducted for the results from the CAM Assay. The Shapiro-Wilk Normality test was also conducted to test the normality of the data from the CAM Assay. The parametric One-way Analysis of Variance (ANOVA), with a 95% confidence level and alpha

value of 0.05, through the R Commander software was used for the statistical analysis of the results, followed by a post hoc test using the Tukey test (Camposano et al., 2016).

RESULTS AND DISCUSSION

Species Identification

The plant species was certified to be *Rubus fraxinifolius* Poir. at the University of the Philippines Diliman Institute of Biology Herbarium.

Extraction

Sapinit fruit was macerated and freeze dried, and a total yield of 22.95 g (1.05%) was produced. Methanol was used for the maceration of sapinit fruit because methanolic extracts of *Rubus* fruits were found to produce the highest phenolic content and anticancer activity in a study done by Muniyandi et al. (2019).

Phytochemical Screening

Qualitative phytochemical screening was conducted on sapinit (*Rubus fraxinifolius*) fruit crude extracts for the presence of the following secondary metabolites: alkaloids, tannins, phenols, free anthraquinones, sterols, flavonoids, and leucoanthocyanins (Figure 1).

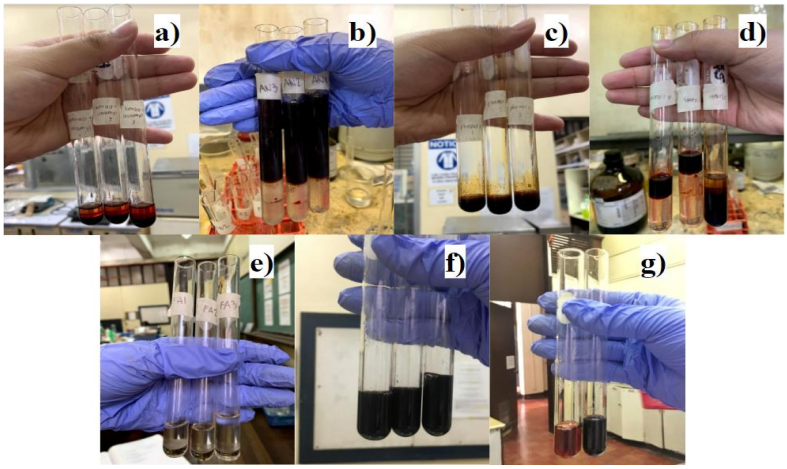


Figure 1. Qualitative Phytochemical tests for a) leucoanthocyanins (+), b) free anthraquinones (-), c) phenols (+), d) sterols (-), e) flavonoids (-), f) tannins (+), and g) alkaloids (-)

The methanolic extracts were tested for these phytochemicals since phenolic compounds are known to have anticancer properties (Dai & Mumper, 2010). Flavonoids and tannins in particular have exhibited antiangiogenic properties (Cai et al., 2016; Gacche, Shegokar, Gond, Yang, & Jadhav, 2011). Through the phytochemical screening, the presence of phenolic compounds that could possibly inhibit angiogenesis in the CAM assay could be detected.

Table 1 shows the results of three trials of phytochemical screening. Tannins were detected through the ferric chloride test, phenols were detected through the ellagic acid test, and leucoanthocyanins were detected through the test for leucoanthocyanins. However, alkaloids were not detected through the Dragendorff's reagent test, free anthraquinones were not detected through the Borntrager's reaction, sterols were not detected through the Salkowski test, and flavonoids were not detected through the ammonium test.

Table 1. Qualitative Phytochemical Screening of Sapinit (*Rubus fraxinifolius*) Fruit Crude Extracts

Phytochemical	Positive Indicator	Results
Alkaloids	Orange precipitate	-
Tannins	Blue-black (hydrolyzable) or brownish-green color (condensed)	+
Phenols	Muddy brown color	+
Free anthraquinones	Layering with pink, red, or violet coloration	-
Sterols	Red chloroform layer and greenish yellow fluorescent acid layer	-
Flavonoids	Yellow ammonia layer and light yellow color	-
Leucoanthocyanins	Appearance of red upper layer	+

Tannins and phenols were also detected in *Rubus rosifolius* fruit crude extracts in a study done by Alvarez et al. (2013). Although anthraquinones were detected in Alvarez et al.'s (2013) and Campbell, McKenzie, Murray, Delgoda, & Bowen-Forbes' (2017) studies on *R. rosifolius* fruit

crude extracts, they were not detected in this study. Flavonoids, which were detected in Campbell et al.'s (2017) study, and alkaloids, which were detected in Alvarez et al.'s (2013) study, were also not detected.

The negative results may be due to low concentrations of the phytochemicals in the *R. fraxinifolius* fruit crude extracts used. In a study by Bakar, Ismail, Isha, & Ling (2016), *R. fraxinifolius* had the lowest phenolic and flavonoid content when compared to fruit crude extracts of other *Rubus* species. In another study, the leaf extracts of *R. fraxinifolius* had lower phenolic content compared to *R. rosifolius* (Desmiaty, Elya, Saputri, Hanafi, & Prastiwi, 2018). Due to this, the phytochemicals may not have been readily detected in the *R. fraxinifolius* fruit crude extracts, especially since qualitative screening was conducted.

Previous studies have also shown significant differences between the bioactivities of *Rubus idaeus* (wild raspberries) grown in different geographic locations (Purgar, Duralija, Voća, Vokurka, & Ercisli, 2012). This may explain the disparities between the results of phytochemical screening for *R. fraxinifolius* as compared to other species.

CAM Assay

The antiangiogenic properties of sapinit fruit crude extracts were tested through *in ovo* CAM assay and photographed as seen in Figure 2. Ten-day-old eggs were used for the CAM assay since peak angiogenic activity from factors such as VEGF-A occurs in earlier stages of development, specifically from the eighth to tenth day of the chick embryo development. The heightened angiogenic activity makes the CAM blood vessel system susceptible to antiangiogenic factors (Luay et al., 2018).

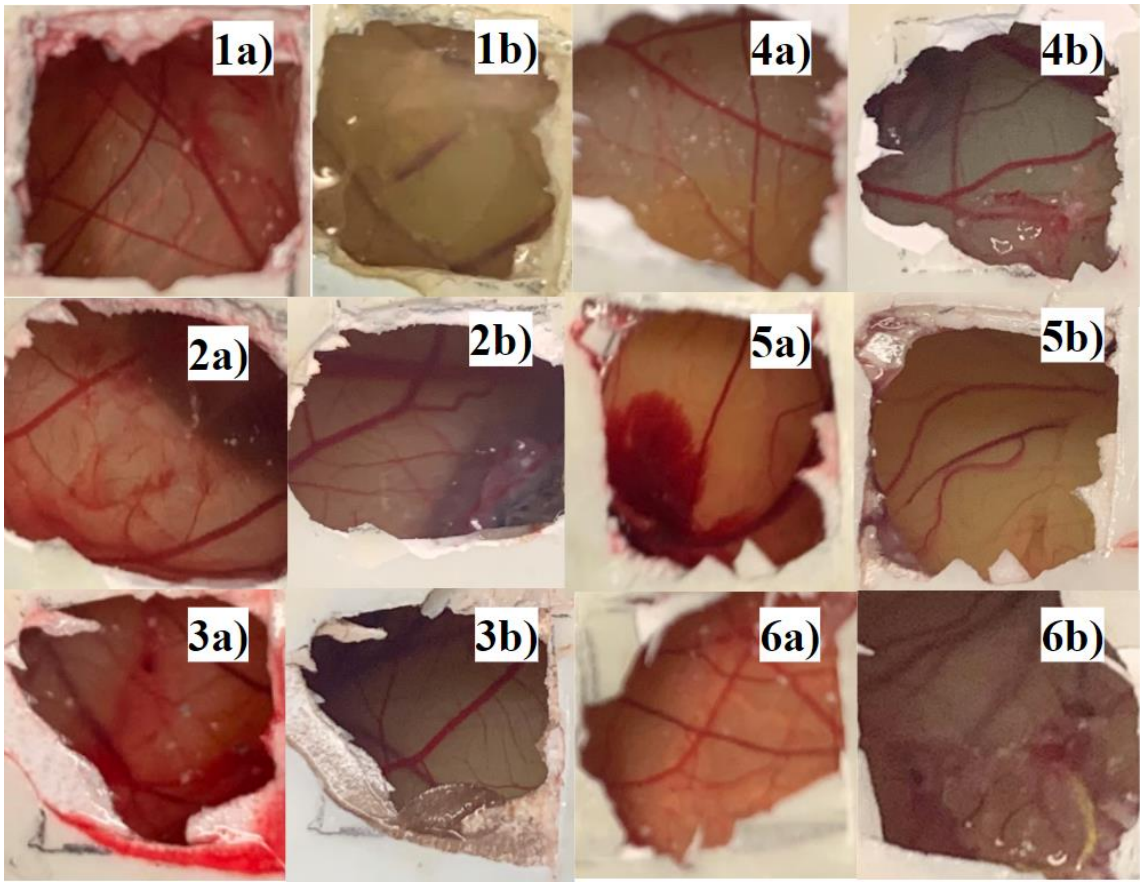


Figure 2. Raw before (a) and after (b) photographs of CAM for the 1) 10 µg/µL treatment, 2) 20 µg/µL treatment, 3) 30 µg/µL treatment, 4) distilled water treatment, 5) methanol treatment, and 6) retinoic acid treatment

The photographs of the CAM were analyzed through ImageJ software (Figure 3). The antiangiogenic properties of the treatments were determined through the percent inhibition of the CAM blood vessels. Total length and vascular density were measured and used as parameters, with positive mean percent inhibitions indicating a decrease in blood vessel growth or antiangiogenesis (Luay et al., 2018).

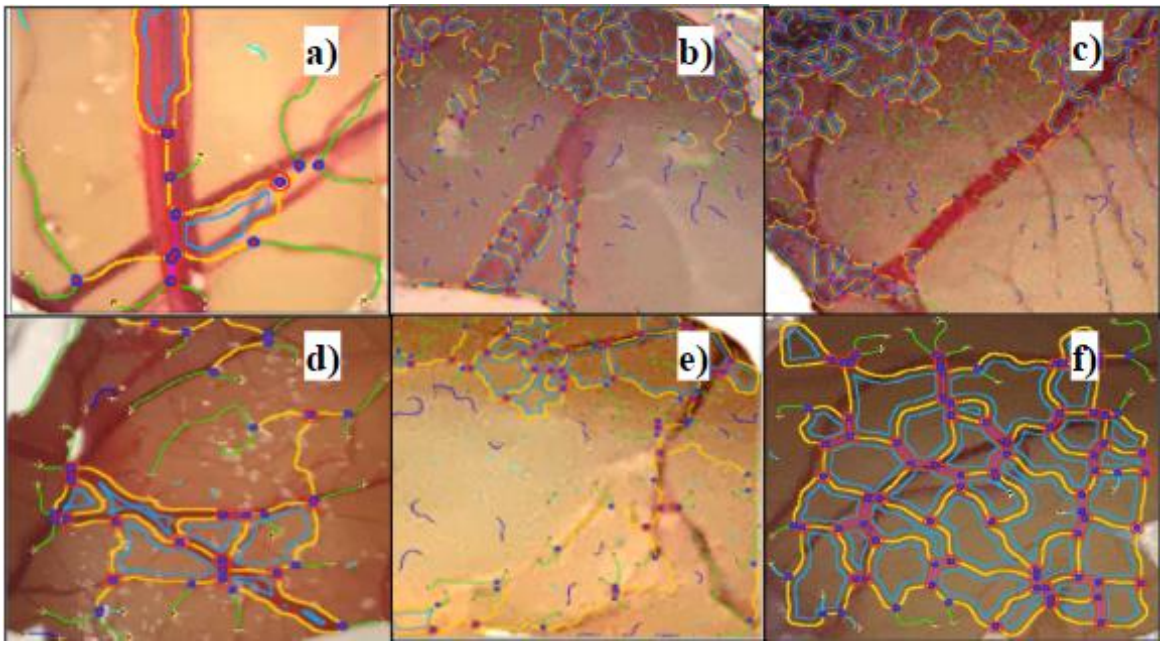


Figure 3. ImageJ software analysis showing human umbilical vein endothelial cell phase contrast of CAM with a) 10 µg/µL treatment, b) 20 µg/µL treatment, c) 30 µg/µL treatment, d) retinoic acid treatment, e) distilled water treatment, and f) methanol treatment

Table 2 shows the mean percent inhibitions of the two parameters after the application of treatments according to the ImageJ Software analysis (Figure 4). For total length, the lowest mean inhibitions were observed for methanol and distilled water, with methanol having a mean inhibition of -13.89% and distilled water having 0.00% mean inhibition. The 10 µg/µL concentration of methanolic extract displayed the highest mean percent inhibition out of the three concentrations and the second highest overall with a value of 118.20%. The 20 µg/µL and 30 µg/µL concentration had a mean inhibition of 3.40% and 5.02% respectively. Lastly, the highest mean inhibition was observed with retinoic acid with a value of 564.97%.

The results on the mean percent inhibition of vascular density were normalized using the arcsine transformation method. The 20 µg/µL and 30 µg/µL concentration had the lowest mean percent inhibition of vascular density, with a mean inhibition of -211.22% and -168.36% respectively. The 10 µg/µL concentration of methanolic extract displayed the highest mean percent

inhibition of vascular density out of the three concentrations and the second highest overall with a value of 24.19%. Similar to the total length parameter, the highest mean inhibition was observed with retinoic acid with a value of 56.39%.

Table 2. Mean Percent Inhibition of the Treatments in the CAM Assay analyzed in ImageJ Software

Treatment	Mean Percent Inhibition (%)	
	Total Length	Vascular Density
Distilled Water	0.00	0.00
Methanol	-13.89	22.44
Retinoic Acid	564.97	56.39
10 μg/μL methanolic extract	118.20	24.19
20 μg/μL methanolic extract	3.40	-211.22
30 μg/μL methanolic extract	5.02	-168.36

The results of the ANOVA Test for the mean percent inhibitions of total length of the blood vessels show that the p-value is 8.3100×10^{-6}. There is a significant difference between the group means of at least one of the pairs of treatments and a post hoc test using the Tukey Test was conducted.

The results of the Tukey Test show that only the difference between the retinoic acid treatment and the rest of the treatments are significant ($p = 0.0010$). There are no significant differences between the treatments with different concentrations and treatments with negative controls ($0.5800 \leq p \leq 1.0000$).

Meanwhile, the results of the ANOVA Test for the mean percent inhibitions of vascular density show that the p-value is 0.1630 and there is no significant difference between the group means of at least one of the pairs of treatments. Both ANOVA Test results have shown that there

is no significant difference between the extract treatments and the negative control treatments, distilled water and methanol.

Although the results from both parameters differ, they both show that the extract treatments do not present significant increases in the inhibition of blood vessel growth.

In Muniyandi et al.'s (2019) study, 1–10 μg/μL concentrations of different *Rubus* fruit extracts exhibited high antioxidant capacity and cytotoxicity. It is possible that the concentrations of *R. fraxinifolius* extract used in the study did not give the desired results since they are not the concentrations needed to achieve optimal antiangiogenic activity. Since the concentrations of *R. fraxinifolius* fruit extracts used in this study were 10, 20, and 30 μg/μL, lower concentrations like in Muniyandi et al.'s (2019) study may be tested in future studies.

The produced results may also be due to the absence of some phytochemicals that exhibit significant antiangiogenic properties such as alkaloids. Although tannins and phenols that possess antiangiogenic properties were detected in the *R. fraxinifolius*, the lack of alkaloids may have greatly affected the antiangiogenic potential (Alasvand et al., 2019). In a study by Camposano et al. (2016), the antiangiogenic activity of 10 μg/μL crude extracts of three plant species tested were also attributed mainly to the presence of alkaloids, as well as sterols, tannins, and other phenolic compounds.

In another study by Zhao et al. (2014), alkaloids were isolated from *Rubus alceifolius* Poir species, and its effects on angiogenesis were evaluated through *in vivo* CAM assay. The total alkaloids in *Rubus alceifolius* Poir inhibited the formation of blood vessels in the CAM assay with a concentration of 100 μg/μL (Zhao et al., 2014). Alkaloids exhibit antiangiogenic activity through various mechanisms (Lopes, Regasini, de Magalhães Pinheiro Alçada, & Soares, 2013). Different types of alkaloids show different mechanisms in inhibiting angiogenesis, and berberine, noscapine,

sanguinarine, and taspine are some examples of those with the most promising inhibitors (Alasvand et al., 2019). These alkaloids inhibit angiogenesis by suppressing vascular endothelial growth factors (VEGF), the main factors of angiogenesis, which are stimulated by hypoxic conditions or conditions wherein there is a lack of oxygen supply needed for blood vessel growth supporting tumor growth (Alasvand et al., 2019). Considering this, isolating certain phytochemicals from the fruit extracts to test for antiangiogenesis may produce better results.

SUMMARY AND CONCLUSION

This study was able to evaluate the antiangiogenic properties of sapinit (*R. fraxinifolius*) fruit crude extract using the chick chorioallantoic membrane (CAM) assay and compare the bioactivities of 10, 20, and 30 μg/μL methanolic extract concentrations. Qualitative phytochemical screening of sapinit fruits was also conducted, in which tannins, phenols, and leucoanthocyanins were detected.

The results of the CAM assay show that although the results for the total length and vascular density parameters differ, they both show that the extract treatments do not present significant increases in the inhibition of blood vessel growth.

Overall, the observed antiangiogenic activity of the test extracts in this study may be attributed to the use of concentrations that may not achieve optimal antiangiogenesis, or low concentration of the phytochemicals, particularly those known to inhibit angiogenesis such as alkaloids, in the extract. Considering this, using lower concentrations of the extract or isolating certain phytochemicals from the *R. fraxinifolius* fruit extracts to test for antiangiogenesis may produce better results.

BIBLIOGRAPHY

Aguinaldo, A., Espeso, E., Guevara, B., & Nonato, M. (2005). *A guidebook to plant screening: Phytochemical and biological*. Manila: University of Santo Tomas Publishing House.

Alasvand, M., Assadollahi, V., Ambra, R., Hedayati, E., Kooti, W., & Peluso, I. (2019). Antiangiogenic Effect of Alkaloids. *Oxidative Medicine and Cellular Longevity, 2019,* 1–16. doi:10.1155/2019/9475908

Alvarez, L.V., Caunca, E.S., & Nolido, J.G. (2013). Antimicrobial activity of *Rubus rosifolius* J.E SM. (Rosaceae) fruit crude extract. *Polytechnic University of the Philippines Journal of Science and Technology, 6*(1), 1-9. Retrieved from https://www.researchgate.net/publication/312580736_ANTIMICROBIAL_ACTIVITY_OF_RUBUS_ROSIFOLIUS_JE_SM_ROSACEAE_FRUIT_CRUDE_EXTRACT

Bakar, M. F. A., Ismail, N. A., Isha, A., Ling, A. L. M. (2016). Phytochemical composition and biological activities of selected wild berries (*Rubus moluccanus* L., *R. fraxinifolius* Poir., and *R. alpestris* Blume). *Evidence-Based Complementary and Alternative Medicine*, *2016*, 1-10. doi:10.1155/2016/2482930

Bansode, T., & Salalkar, B. K. (2015). Phytochemical analysis of some selected Indian medicinal plants. *International Journal of Pharma and Bio Sciences, 6*(1), 550-556.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians 2018, 68,* 394-424. doi:10.3322/caac.21492

Cai, Y., Zhang, J., Chen, N. G., Shi, Z., Qiu, J., He, C., & Chen, M. (2016). Recent advances in anticancer activities and drug delivery systems of tannins. Medicinal Research Reviews, 37(4), 665–701. doi:10.1002/med.21422

Campbell, T. F., McKenzie, J., Murray, J., Delgoda, R., & Bowen-Forbes, C. S. (2017). *Rubus rosifolius* varieties as antioxidant and potential chemopreventive agents. *Journal of Functional Foods*, *37*, 49–57. doi:10.1016/j.jff.2017.07.040

Camposano, J. E., Dela Torre, G. L. T., Laxamana, J. G , & Larcia, L. L. H., II. (2016). Screening for the anti-angiogenic activity of selected Philippine medicinal plants using chorioallantoic membrane assay. *Mahidol University Journal of Pharmaceutical Sciences, 43*(4), 173-182. Retrieved from https://www.pharmacy.mahidol.ac.th/journal/_files/2016-43-4_2.pdf

Dai, J., & Mumper, R. J. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules, 15(10), 7313–7352. doi:10.3390/molecules15107313

Department of Health. (n.d.). *What are the leading causes of death in the Philippines?* Retrieved September 12, 2019, from https://www.doh.gov.ph/node/1058

Desmiaty, Y., Elya, B., Saputri, F. C., Hanafi, M., & Prastiwi, R. (2018). Antioxidant activity of *Rubus fraxinifolius* Poir. and *Rubus rosifolius* J. Sm. leaves. *J Young Pharm*, *10*(2), s93-s96. Retrieved from https://www.jyoungpharm.org/sites/default/files/JYoungPharm_10_2_s93.pdf

Gacche, R. N., Shegokar, H. D., Gond, D. S., Yang, Z., & Jadhav, A. D. (2011). Evaluation of selected flavonoids as antiangiogenic, anticancer, and radical scavenging agents: An experimental and in silico analysis. Cell Biochemistry and Biophysics, 61(3), 651–663. doi:10.1007/s12013-011-9251-z

Hjartåker, A., Knudsen, M.D., Tretli, S., & Weiderpass, E. (2014). Consumption of berries, fruits and vegetables and mortality among 10,000 Norwegian men followed for four decades. *European Journal of Nutrition*, *54*(4), 599-608. doi:10.1007/s00394-014-0741-9

International Agency for Research on Cancer. (2018). *Estimated number of new cases in 2018, Philippines, both sexes, all ages*. Retrieved September 12, 2019, from http://gco.iarc.fr/today/online-analysis-table?v=2018&mode=cancer&mode_population=continents&population=900&populations=608&key=asr&sex=0&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&nb_items=5&group_cancer=0&include_nmsc=1&include_nmsc_other=1

Jimenez-Garcia, S. N., Vazquez-Cruz, M. A., Garcia-Mier, L., Contreras-Medina, L. M., Guevara-González, R. G., Garcia-Trejo, J. F., & Feregrino-Perez, A. A. (2018). Phytochemical and pharmacological properties of secondary metabolites in berries. *Therapeutic Foods, 8,* 397-427. doi:10.1016/b978-0-12-811517-6.00013-1

Le, P. T. Q. (2019). Phytochemical screening and antimicrobial activity of extracts of *Cassia alata* L. leaves and seeds. *Bulgarian Chemical Communications, 51*(3), 378-383. doi:10.34049/bcc.51.3.5049

Lopes, F. C. M., Regasini, L. O., de Magalhães Pinheiro Alçada, M. N., & Soares, R. (2013). *Antiangiogenic Alkaloids from Plants. Natural Products, 1439–1467.* doi:10.1007/978-3-642-22144-6_48

Luay, M. A. M., Gonzaga, M. F. R., Po, S. K. D., & Arollado, E. C. (2018). Determination of the antiangiogenic activity of *Telescopium telescopium* (horn snail) extract using in ovo chorioallantoic membrane (CAM) assay. *ACTA MEDICA PHILIPPINA, 52*(4), 366-373. Retrieved from https://www.actamedicaphilippina.org/api/v1/articles/5142-determination-of-the-antiangiogenic-activity-of-telescopium-telescopium-horn-snail-extract-using-in-ovo-chorioallantoic-membrane-cam-assay.pdf

Muniyandi, K., George, E., Sathyanarayanan, S., George, P. B., Abrahamse, H., Thamburaj, S., & Thangaraj, P. (2019). Phenolics, tannins, flavonoids and anthocyanins contents influenced antioxidant and anticancer activities of *Rubus* fruits from Western Ghats, India. *Food Science and Human Wellness, 8*, 73-81. doi:10.1016/j.fshw.2019.03.005

Ngelangel, C. A., Lam, H. Y., Rivera, A. S., Kimman, M. L., Real, I. O., Balete, S. L., and Philippines ACTION Study Group. (2018). Philippine Costs in Oncology (PESO): Describing the economic impact of cancer on Filipino cancer patients using the ASEAN costs in oncology study dataset. *Acta Medica Philippina*, *52*(2), 125-133.

Philippine Statistics Authority. (2019). *Vital statistics 2018 - Preliminary counts of births, marriages and deaths*. Retrieved September 12, 2019, from https://psa.gov.ph/civilregistration/vital-statistics-2018-preliminary-counts-births-marriages-and-deaths

Purgar, D. D., Duralija, B., Voća, S., Vokurka, A., & Ercisli, S. (2012). A comparison of fruit chemical characteristics of two wild grown *Rubus* species from different locations of Croatia. *Molecules*, *17*, 10390-10398. doi:10.3390/molecules170910390

Rodriguez-Saona, L. E., & Wrolstad, R. E. (2001). Extraction, Isolation, and Purification of Anthocyanins. In *Current Protocols in Food Analytical Chemistry*. John Wiley & Sons, Inc.

Sapinit: Philippine Wild Raspberry. (2018). Retrieved from https://businessdiary.com.ph/2656/sapinit-philippine-wild-raspberry/

Sheel, R., Nisha, K., & Kumar, J. (2014). Preliminary phytochemical screening of methanolic extract of *Clerodendron infortunatum. IOSR-Journal of Applied Chemistry, 7*(1), 10-13. doi:10.9790/5736-07121013

Tantiado, R. G., & Tan, V. P. (2012). Evaluation of the angiosuppresive activity of *Tinospora rumphii* Boerl. stem extract using the chorioallantoic membrane assay in *Anas platyrhynchos* embryos. *International Journal of Bio-Science and Bio-Technology, 4*(2), 93-102. Retrieved from http://modul.repo.mercubuana-yogya.ac.id/modul/files/openjournal/JournalOfInformation/9_387.pdf

World Health Organization. (2018). *Cancer*. Retrieved from https://www.who.int/news-room/fact-sheets/detail/cancer

Zhao, J., Lin, W., Zhuang, Q., Zhong, X., Cao, Z., Hong, Z., & Peng, J. (2014). *Total Alkaloids of Rubus alceifolius Poir Shows Anti-Angiogenic Activity In Vivo and In Vitro. Integrative Cancer Therapies, 13(6), 520–528.* doi:10.1177/1534735414547109

【評語】080019

Rubus fraxinifolius or “sapinit” has been found to possess phytochemicals with anti-cancer potential. This project aimed to evaluate the antiangiogenic properties of methanolic R. fraxinifolius fruit crude extracts through the chick chorioallantoic membrane (CAM) assay. Through aualitative phytochemical screening, leucoanthocyanins, phenols, and tannins were detected.

Strong points:
CAM is a typical way to assay for the antiangiogenic properties. Through their study, retinoic acid treatment has a significantly higher mean percent inhibition (MPI) based on the results of the Tukey Test. The writing of this report was followed by the citation of proper scientific references.

Weakness:
The results for the MPI of vascular density show no significant differences between all groups. They comment that it is possible that the concentrations used in the study may not reach the concentrations needed to achieve optimal antiangiogenesis. The results may also be due to the absence of phytochemicals that exhibit significant antiangiogenic properties such as alkaloids. The goal for finding antiangiogenic compounds from methanolic R. fraxinifolius fruit crude extracts is not reached. In conclusion, they comment that using lower concentrations of the extract or

isolating certain phytochemicals from the R. fraxinifolius fruit extracts to test for antiangiogenesis may produce better results. Should it be using higher concentration of the extract? Did they try?

Research Comments:

1. The writing of this report was followed by the citation of proper scientific references. Your research methods mentioned the use of proper statistical comparisons in Table 2, but lack of enough replicates to validate your results. Although the COVID-19 pandemic limits your research, it is recommended that more data are required in order to complete this high-end project conducted by high school students in future.
2. Acidified methanol was used to extract potential antiangiogenic contents in this project. Eighty % methanol extracts were evaluated for antioxidant and antibacterial activities in Bakar et al. (2016) in your Bibliography. Is that possible your potential antiangiogenic contents exist in water extracts, but not in organic phase? For example, tannins and alkaloids are water soluble.