

COMBINING *IN VITRO* AND *IN OVO* ASSAYS TO SCREEN FOR ANTI-CANCER AND ANTI-ANGIOGENIC EFFECTS OF THE LEAF EXTRACTS OF *Mallotus cumingii* Müll.Arg. (EUPHORBIACEAE)

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ABSTRACT

Cancer treatment is often challenging and various interventions may have detrimental effects. Due to this, the development of less harmful alternatives such as herbal medicine is essential. The present study aims to determine the leaf phytoconstituents present and the bioactivities of *Mallotus cumingii* Müll.Arg against cancer cells through the utilization of MTT assay and anti-angiogenesis through CAM assay. The leaf extracts obtained three fractions namely, methanolic crude (MCME) extracts, hexane extracts (MCHE), and ethyl acetate extracts (MCEA), and was tested on HCT-116 for *in vitro* cytotoxicity, and blood vessel density and branching through *in ovo* CAM assay. Phytochemical analysis showed that the *M. cumingii* fractions contain phenolic compounds, terpenoids, cardiac glycosides, flavonoids, and saponins. For *in vitro* set-up, MCME of *M. cumingii* were separated into MCHE and MCEA partitions and were tested against HCT-116 and obtained an IC₅₀ value of < 30 µg/mL, which is deemed active in cytotoxicity. For *in ovo* set-up, two concentrations of each extract were applied to the duck eggs. Blood vessel density and number of branching points were measured through the ImageJ analysis. All extracts exhibited anti-angiogenic activity, either by decreasing blood vessel density or the number of branching points. Overall, the study demonstrates the potential of *M. cumingii* as a source of therapeutic agents.

Key words: Anti-angiogenic, anti-cancer, Philippines, phytochemical analysis

INTRODUCTION

In 2020, it was estimated that there were 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred worldwide with colorectal cancer determined to be the third most common cancer (1,800,000 cases, 10.0% of the total) and the second most common cause of mortality (862,000 deaths, 9.4% of the total) (Bray *et al.*, 2018). In Asia, the mortality due to colorectal cancer has been increasing in the last decade in Asian countries, except in Japan and Singapore (Hyodo *et al.*, 2010). In a study published by Arnold *et al.* (2017), countries were grouped based on the trends of incidence and mortality of colorectal cancer. The Philippines was placed under the first group, where increases in both incidence and mortality have been observed since 2006. As of 2020, the incidence of colorectal cancer in the Philippines was determined to be the 3rd leading site of malignancy in the country, after the cancers of the breast and the lung (Ting *et al.*, 2020). In the light of these alarming statistics, it is no wonder that much focus is put into cancer research. According

to Cancer Research United Kingdom (2020), £455 million have been spent on studying the prevention, diagnosis, and treatment of cancer in 2019/2020.

Cancer cells can rapidly divide to form tumors, which are designated as benign if they do not invade surrounding tissues; in the case that these do, they are classified as malignant (Patel, 2020). Malignant tumors can trigger a new formation of blood vessels from pre-existing vessels termed as angiogenesis (Rodriguez *et al.*, 2021). This process plays an important role in various physiological and developmental processes such as embryonic blood vessel formation, wound healing, and menstrual cycle (Rajabi *et al.*, 2017). However, cancer cells hijack angiogenesis and this allows for the formation of blood vessels that permits tumor cells to enter the bloodstream and spread to different organs of the body in a process called metastasis (Hicklin & Ellis, 2005). The lack of angiogenic phenotype is directly related to the low metastatic activity of tumors (Folkman, 2002).

Nature has long been a source of treatment in the field of medicine. According to Farnsworth *et al.* (1985), the World Health Organization (WHO) estimated in 1985 that around 65% of the world's

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population rely mainly on plant-derived traditional medicines as a source of primary health care. Hartwell (1970) listed more than 3000 plant species that have been reportedly used in the treatment of cancer. Many effective anticancer agents that are used today were extracted from plants, such as Taxol (Paclitaxel), Taxotere (Docetaxel), and Vincristine (Mukherjee *et al.*, 2001). Medicinal plants often contain various phytochemicals that have different effects and purposes. The most common are flavonoids, phenols, and terpenes (Doughari *et al.*, 2012). Flavonoids interfere with angiogenesis by disrupting the basement membrane of blood vessels (Ren *et al.*, 2003), while the polyphenol rottlerin in *Mallotus philippinensis* inhibits the expression of NF- κ B transcription factors that are necessary for angiogenesis (Sharma, 2011). One potential plant that may contain anti-angiogenic compounds is *Mallotus cumingii*. It is also known as *Neotrewia cumingii* and belongs to the family Euphorbiaceae. *M. cumingii*, commonly called Apanang, is found mostly in the Philippines, and its distribution extends to Borneo and Sulawesi. In the Philippines, *M. cumingii* is widely distributed in Luzon in the Tayabas province (Kulju *et al.*, 2007). Its narrow geographic range makes it a good target for research in tropical countries including the Philippines. Plants from the family Euphorbiaceae have been widely studied for their cytotoxic activities against cancer cell lines (Aliomrani *et al.*, 2017). Plant extracts from this family are commonly used as treatments for ulcers, cancer tumors, and warts among other diseases (Betancur-Galvis *et al.*, 2002). A closely related species, *M. philippinensis*, has been studied extensively and its fruits have shown anticancer activity against human cancer cell lines (Sharma, 2011). Contrarily, the anticancer, antiangiogenic, and general bioactivity of *M. cumingii* has not yet been investigated.

The study aims to contribute to the screening of anti-cancer drug candidates by investigating the cytotoxicity and anti-angiogenic properties of *M. cumingii* crude extract, hexane, and ethyl acetate partitions. MTT assay was used as an *in vitro* model of cytotoxicity to cancer cells while CAM assay was used as an *in ovo* model of angiogenesis. The cytotoxic activities of the extracts were used to display active cytotoxic properties against HCT-116 (human colorectal cancer cell line). The anti-angiogenic activity of the crude extract and two partitions are compared to determine which contains the anti-angiogenic phytochemicals. Phytochemical analysis was performed to identify the phytochemicals present in the extracts.

MATERIALS AND METHODS

Plant collection and extraction

Samples of *M. cumingii* were collected from Mt. Makiling, Los Baños, Laguna, Philippines. Samples were brought to the Jose Vera Santos Memorial Herbarium (PUH), Institute of Biology, University of the Philippines- Diliman, for verification and proper identification of specimens that were subsequently

deposited under accession #1257. After identification, the leaves were left to air dry for two weeks. Then, the dried leaves were macerated into powder-like form and placed in a jar. Methanol was then placed in the jar containing the macerated leaves and the leaves were left soaked for 48 h. The resulting suspension was filtered and concentrated using a rotary evaporator at 37 °C. A crude extract was obtained and air-dried (Rodriguez *et al.*, 2021).

Hexane and ethyl acetate solvent partitioning

The crude extract was exhaustively partitioned using hexane and ethyl acetate. 10 mg of methanolic crude extract was dissolved in 150 mL distilled methanol and was transferred into a 500 mL separatory funnel. 100 mL deionized water was added into the separatory funnel. Afterward, 250 mL of n-hexane was added to the funnel. The solution was mixed and was left to stand until the two layers were observed (hexane partition at the upper layer and methanol partition at the bottom). The hexane layer was collected and subjected to rotary evaporation. The process was repeated until the hexane layer turned colorless. The extract obtained from rotary evaporation was collected and air-dried. The remaining aqueous layer was used for the ethyl acetate partitioning. 100 mL of deionized water, followed by 250 mL of ethyl acetate was added into the separatory funnel. The set-up was left standing until the aqueous and organic-ethyl acetate layers were formed. The two layers were separated into different containers. The ethyl acetate partition was subjected to rotary evaporation. This was repeated until the ethyl acetate layer turned clear. After this, the extract was transferred into a vial and air-dried (Dapat *et al.*, 2013).

Phytochemical analysis

Crude, hexane, and ethyl Acetate extracts were subjected to phytochemical screening based on the method from Harborne (1998) and Onwukaeme *et al.* (2007) with modifications. The screening was conducted for each extract to determine the presence of secondary metabolites mentioned below. Three trials were performed for each phytochemical test.

Detection of saponins

1 mg/mL of extract in distilled water was boiled, followed by cooling, and shaking of the cooling solution. Frothing of the solution indicates the presence of saponin in the extract.

Detection of flavonoid

2 mg of extract was dissolved using 1.0M NaOH. The solution should change color from yellow to orange. Then, 1.0 M HCl was added dropwise to the solution. A yellow to orange coloration of the solution that turns colorless upon the addition of HCl indicates the presence of flavonoids.

Detection of phenols

1% FeCl₃ was added dropwise to 1 mg/mL of extract in distilled water. Transformation of the solution to green, blue, black, or purple color, indicates that phenol is present in the extract.

Detection of tannins

15% FeCl₃ solution was added dropwise to 0.4 mg/mL of extract in distilled water. The formation of a blue-black precipitate indicates the presence of hydrolyzable tannins while a brownish-green precipitate indicates the presence of condensed tannins.

Detection of terpenoid

Concentrated sulfuric acid (H₂SO₄) was added dropwise to 1 mg/mL of extract in chloroform. The formation of a reddish-brown interface indicates the presence of terpenoids.

Detection of cardiac glycoside

1% FeCl₃ was added to 1mg/mL of extract in distilled water. Then 1 mL of concentrated sulfuric acid (H₂SO₄) was added to the solution. The formation of a brown ring indicates the presence of cardiac glycosides in the extraction.

In vitro cytotoxicity (anti-cancer) assay

Cell culture maintenance

Human colorectal cancer cells (HCT-116) were grown in a Corning T-flask (containing 87.5% Modified McCoy's 5A Medium with L-glutamine, 10.0% Fetal Bovine Serum (FBS), 1.0% anti-microbial anti-mycotic (AA) solution, and 1.5% sodium bicarbonate) and were washed regularly to replace spent media and discard dead cells. Passaging was done on cells once they have reached at least 90% confluence in the culture flasks. To detach cells from the flask, the cells were exposed to trypsin-EDTA. Fresh media were added to neutralize trypsin-EDTA after all the cells have already been detached. Most of the cells in the media were discarded, save for one to two drops that were retained in the flask. A fresh medium was then added, and the remaining cells were allowed to grow.

MTT assay

The MTT cytotoxicity assay performed in this study was adapted from Mosmann (1983). The HCT-116 cells were seeded at 4 × 10⁴ cells/mL in sterile 96-well microtiter plates and incubated overnight at 37 °C and 5% CO₂ and 95% humidity. Eight two-fold dilutions of the sample were used as treatments starting from 100 µg/mL down to 0.78125 µg/mL. Cells treated with Doxorubicin served as positive control and treatment with dimethyl sulfoxide (DMSO) as a negative control. After treatment, cells were incubated again for 72 h at 37 °C and 5% CO₂. The media were then removed and replaced with 20 µL of 5 mg/mL MTT dye dissolved in phosphate buffer solution (PBS) in each well. The cells were incubated once again at 37 °C and 5% CO₂ for 4 h. DMSO at 150 µL, was then added to all wells before the cells' absorbance was read at 570 nm using a LEDTECT 96-well plate reader. The percent inhibition per concentration was calculated using formula as in Equation 1:

$$\text{Inhibition (\%)} = \left(\frac{\text{optical density of DMSO} - \text{optical density of sample}}{\text{optical density of DMSO}} \right) \times 100$$

- Equation 1

The Inhibition Concentration 50 (IC₅₀) was computed from the inhibition values against the concentration of the sample/controls using GraphPad Prism 7. Three trials were done for every assay. Samples with IC₅₀ values less than 30 µg/mL were considered active (Jokhadze *et al.*, 2007).

In ovo anti-angiogenic assay

Treatment preparation

From the crude extract, a stock solution was made by dissolving 100 mg of crude extract into 10 mL of dimethyl sulfoxide (DMSO). To prepare the 0.4 mg/mL (high) concentration, 0.6 mL of the stock solution was mixed with 14.4 mL 1× PBS. Then, 0.06 mL of stock solution was added to 14.94 mL of 1× PBS and 0.54 mL of DMSO to prepare the 0.04 (low) mg/mL treatment. The stock solution for both hexane and ethyl acetate treatments was made similarly to the crude extract stock solution. To make the hexane stock solution, 100 mg of hexane partition was dissolved into 10 mL of DMSO. To prepare the hexane high concentration, 0.6 mL of stock solution was mixed with 14.4 mL 1× PBS. Then, 0.06 mL of stock solution was added to 14.94 mL of 1× PBS and 0.54 mL of DMSO to prepare the low treatment. To make the ethyl acetate stock solution, 100 mg of ethyl acetate partition was dissolved into 10 mL of DMSO. Then the hexane high concentration was made, 0.6 mL of stock solution was mixed with 14.4 mL 1× PBS. Then, 0.06 mL of stock solution was added to 14.94 mL of 1× PBS and 0.54 mL of DMSO to prepare the ethyl acetate low treatment. For the positive control, 50,000 IU Retinoic Acid was dissolved in DMSO. From this solution, 0.6 mL was obtained and diluted in 14.4 mL PBS. Similarly, 0.6 mL DMSO was diluted with 14.4 PBS to prepare the negative control (Rodriguez *et al.*, 2021).

CAM assay

Day 8 *Anas platyrhynchos* eggs were obtained and surface sterilized with 75% ethanol. The eggs were then placed in incubation at 37 °C. After 2 days of incubation, the eggs were candled using the candle chamber to see and mark the embryo's position. After this, a small opening was made at the airspace in sterile conditions and treated with 200 µL of *M. cumingii* crude extract of 0.4 mg/mL concentrations. The opening was then sealed with parafilm after treatment. The egg was then left to stand for thirty min before laying it on its side. After this, the egg was incubated at 37 °C. The previous steps were repeated with the 0.04 mg/mL treatment. A positive and negative control setup was also performed using retinoic acid and PBS with DMSO, respectively. After two days, the egg was windowed where the embryo can be found, and a picture of the CAM was taken at this stage (CAM_{data}). The CAM assay was repeated for hexane and ethyl acetate treatments. The pictures from all treatments were processed by tracing the blood vessels using Adobe Photoshop and then analyzed using the software ImageJ. The blood vessel density was assessed as the percent area fraction, which is the percent of traced blood vessel pixels relative to the total pixels of the CAM multiplied to 100%, and by counting the blood branching points (Rodriguez *et al.*,

2021). This method would result to separate data for branching points and total blood vessel density since changes in angiogenesis may occur through one or both mechanisms.

Data and statistical analysis

In the *in vitro* setup, the computation of IC_{50} values of the treatments was carried out using GraphPad Prism 7. In the *in ovo* set up, LOGIT transformation was performed with percent area fraction data then, the number of branching points and percent area fraction means were compared between treatment groups. For both set-ups, Levene's test for homogeneity and Shapiro-Wilk test for normality was used before One-Way Analysis of Variance (ANOVA) via the IBM SPSS Statistics 22 software. Subsequently, Tukey's Honest Significant Difference (HSD) test was used for *in vitro* set up while, Least Significant difference tests were performed on the *in ovo* set up to see if there are significant changes between the number of branching points and percent area fraction at different treatments. The concentration with a significant effect was determined. *P-values* < 0.05 indicated significant differences between the means that were compared.

RESULTS AND DISCUSSION

Phytochemical screening of crude, hexane, and ethyl acetate extracts

Phytochemical screening of the three solvent partitions of *M. cumingii* (MC) shows that in all three, phenolic compounds were present, indicated by the solution turning green. The majority of the phytochemicals tested were present in crude extract, including terpenoids, flavonoids, cardiac glycosides, and phenols. This was followed by ethyl acetate, which contains saponins, flavonoids, and phenols as well. Meanwhile, hexane only had phenolic compounds (Table 1). In the present study, the extracts, through solvent partitioning, allow different phytoconstituents to be extracted by the varying

polarity of each solvent used. Methanol was used for crude extraction due to its high polarity. The crude extracts from methanolic extraction were expected to contain higher numbers of phenolic compounds. This was followed by hexane partitioning. Hexane is a non-polar solvent and allows non-polar compounds such as terpenoids to be extracted (See *et al.*, 2017). Results from phytochemical screening do not agree with this since terpenoid, typically non-polar, was found in the polar crude extract. However, according to Jiang *et al.* (2016), modifications such as hydroxylations and glycosylations, allow terpenoids to have a more polar structure. In such cases, polar terpenoids may be present in the plant and can be extracted by polar solvents such as methanol. Besides terpenoids, cardiac glycosides were found only in the crude extract. Glycosides are polar compounds that possess anticancer and antioxidant activities (Widyawati *et al.*, 2014; See *et al.*, 2017). Cardiac glycosides are constituents typically used for cardiac conditions such as digoxin. Cardiac glycosides prevent angiogenesis through the regulation of fibroblast growth factor-2 (FGF-2), which promotes angiogenesis, by inhibiting Na-K ATPase activity. Other than this, they also inhibit NF- κ B activation thus inhibiting tumorigenesis (Winnicka *et al.*, 2006). The extract was further partitioned using ethyl acetate and is expected to have compounds with the polarity between methanol and hexane (Tolentino *et al.*, 2021). Based on phytochemical screening, flavonoid, typically a polar compound (Jia *et al.*, 2009), was found in both crude extract and ethyl acetate fraction. On the other hand, saponin was found only in the ethyl acetate fraction. Saponins are typically polar, but some have low polarity (Jia *et al.*, 2009). In a study by Qian *et al.* (2014), low polarity saponins from leaves of *Panax notoginseng* had higher cytotoxic activity than high polarity saponins. Saponins can also inhibit proteases and disrupt phosphorylation of receptors, intervening information of endothelial tubes (Ahmad *et al.*, 2016).

Table 1. Phytochemical constituents present in various extracts

Phytochemicals	Crude	Hexane	Ethyl Acetate
Tannin	Absent	Absent	Absent
Saponin	Absent	Absent	Present
Terpenoids	Present	Absent	Absent
Flavonoids	Present	Absent	Present
Cardiac Glycosides	Present	Absent	Absent
Phenolic compounds	Present	Present	Present

M. cumingii leaf extracts have varying cytotoxic effects on HCT cancer cells

The potential anticancer activity of the MC leaves was tested against the HCT-116 cancer cell line. Different partitions of the leaves were assessed, the crude methanolic extract, hexane, and ethyl acetate partitions. Figure 1 shows the half-maximal inhibitory concentration (IC_{50} , μ g/mL) of these partitions. The results show that both hexane and ethyl acetate fractions of MC were cytotoxic, with

mean IC_{50} values of 17.49 μ g/mL and 7.75 μ g/mL, respectively. The MC crude extract was found to be inactive, but its IC_{50} was considerably near 30, which was 31.51 μ g/mL. Only the IC_{50} of the MC hexane partition as shown in Figure 1 was found not to be normally distributed. However, all three samples were not found to be statistically different from Doxorubicin via Tukey-HSD. According to the protocol of the American Cancer Institute (NCI), IC_{50} values \leq 30 μ g/mL are considered significant for

crude extracts of plant origins while IC_{50} values ≤ 4 $\mu\text{g/mL}$ are significant for pure substances (Guedes *et al.*, 2013). It then stands to follow that the crude methanolic leaf extracts were inactive in cytotoxicity, albeit still being close. However, both the hexane and ethyl acetate partitions obtained from the methanolic extract showed IC_{50} values ≤ 30 $\mu\text{g/mL}$ against HCT-116 cells. The reduced activity in the crude extract of

MC could likely be attributed to antagonistic effects of substances that are needed to be separated to work efficiently. Some possible mechanisms of this antagonistic interaction are the mutual interference of two or more compounds on the same target, or the attack on different targets of related pathways (Jia *et al.*, 2009).

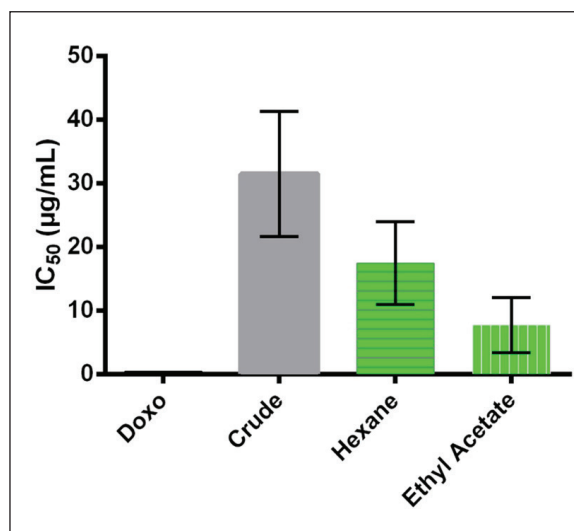


Fig. 1. Half-maximal inhibitory concentration (IC_{50} , $\mu\text{g/mL}$) of the crude methanolic extract, hexane, and ethyl acetate partitions against HCT 116. Values were reported as the mean of three independent tests, each with two replicates. The standard error of the mean (SEM) as shown in the figure as error bars. Fractions deemed active in cytotoxicity (< 30 IC_{50} values) were shown in green.

M. cumingii crude extract significantly reduced blood vessel density

The percent area fraction is the value of the traced blood vessel pixels relative to the total pixels present in the picture multiplied by 100%. The percent area fraction represents the blood vessel area in the CAM and is dependent on the diameter size of the blood vessels. Figure 2 and Table 2 represent the percent area fraction data obtained from CAM in all treatments. As shown in this figure, the average CAM blood vessel density of the negative control was higher than that of all the other treatments. The blood vessel density of CAM treated with the MC methanolic extracts was significantly reduced. During angiogenesis, old blood vessels create new blood vessels by forming new branches. The branching points of CAM in the negative control remain high (Figure 2f), while the positive control exhibited a significant reduction in branching points. The CAM treated with MC methanolic extracts showed a slight reduction but there was no significant difference from the negative control group. Overall, this data suggests that the MC methanolic extract was able to reduce the thickness of blood vessels but not the branching of blood vessels.

It was observed that both crude extract concentrations significantly reduced blood vessel density. Blood vessel density represents the area covered by blood vessels in the chorioallantoic membrane, which may be affected by blood vessel diameter size. The crude extract contained cardiac glycosides and terpenoids, that were not present

in any other fraction, together with phenols and flavonoids. It may be due to this that the crude extract was more effective in lowering blood vessel density. In endothelial cells, NF- κ B pathway promotes angiogenesis inhibition and immune responses (Tabruyn *et al.*, 2008). Blood vessel size is controlled by the angiopoietin-tie2 signaling pathway (Thurston & Daly, 2012). Ang1 is thought to induce an increase in vessel size, genetic deletion studies of its gene show smaller diameter in blood vessels. Compared to other vascular growth factors, Ang1 promotes vessel diameter increase without branching. Blood vessel size can be reduced through inhibition of angiopoietin1 or its tyrosine kinase receptor, Tie2 (Hansen *et al.*, 2010). In a study by He *et al.* (2009), terpenoids from *Tripterygium wilfordii* prevented angiogenesis by reducing angpt2 and tie2 expression. Terpenoids in MC may have had a similar function to reduce blood vessel diameter and eventually, blood vessel density. Other than this, flavonoids and phenols inhibit VEGF activity. VEGF induces the release of Nitric Oxide (NO), a potent vasodilator. NO induces vasodilation through mitogen-activated kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) (Rajabi *et al.* 2017).

In connection to the *in vitro* set up of the crude, the extract showed a non-cytotoxic effect to the chosen cell line but was found to be effective in reducing blood vessel density in CAM *in ovo*. One possibility for the non-cytotoxic nature of the crude to the cell line is the choice of the *in vitro* model

used. According to Franco *et al.* (2016), cancer cell lines do not represent the primary tumor formation and disease progression but may offer promise for

early mechanisms of progression and development of cancers. It is not a single disease, and each known cancer has its reaction to a certain anti-cancer agent.

Table 2. Percent area fraction of the crude extracts representing the mean blood vessel density and branching points with standard error

Treatment	CAM _{data}	
	Blood vessel density	Branching points
Negative	18.624 ± 0.9581	55.2 ± 1.5343
Positive	16.646 ± 0.8288	41.286 ± 3.9341
0.04 mg/mL	12.762 ± 0.5334	52.313 ± 4.4839
0.4 mg/mL	13.162 ± 0.4771	53.118 ± 3.1070

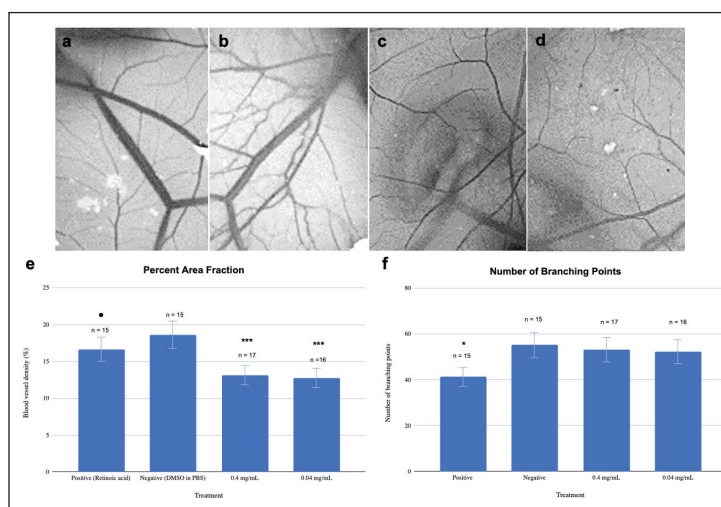


Fig. 2. Images of Egg from control and crude extract set-ups. Shown were sample images of: (a) positive treatment (b) negative treatment; (c) 0.04 mg/mL treatment; (d) 0.4 mg/mL treatment. (e) represents the percent area fraction of the control and crude extract treatments. (f) represents the number of branching points in control and crude extract treatments. ● $p < 0.1$; * $p < 0.05$, significant difference compared to negative control.

***M. cumingii* Hexane and Ethyl Acetate partitions differentially affects blood vessel density and branching**

Blood vessel density, represented by percent area fraction, was significantly reduced in the positive control (Figure 3). Although both hexane and ethyl acetate partitions of MC were able to decrease blood vessel density, only the lower hexane concentration showed a statistically significant reduction. For angiogenesis, the MC hexane partition was able to significantly reduce the number of branching points (0.04 mg/mL). Similarly, MC ethyl acetate partition was also able to reduce the branching of blood vessels but was observed to be highly cytotoxic to CAM and egg (Figure 3h). This was evident by the decreased number of surviving eggs, 27.7% and 22.2% for low and high concentrations, respectively. This suggests that the MC hexane fraction was able to effectively reduce blood vessel density and branching without being toxic to the CAM and growing embryo in the egg.

The ethyl acetate extract was effective in MTT assay but seems to be toxic *in ovo*. This may be attributed to the presence of saponins in the extract. According to Jiang *et al.* (2016), saponins were responsible for most of the toxicity (85.1%-93.6%) in the plant *Quillaja saponaria* when tested to aquatic

crustacean *Daphnia magna* and *Danio rerio* zebrafish embryos. Degradation of the extracted saponins into by-products is effective in making it less toxic.

Among the three extracts, hexane extract was the most effective as anti-angiogenic and anti-cancer due to the presence of the phenolic compounds. Hexane was able to show cytotoxic effects to the chosen cell line and has effectively reduced blood vessel density and branching without being toxic to the CAM or the growing embryo in the egg. Phenolic compounds display cytotoxic anti-cancer agents by promoting apoptosis, reducing tumor proliferation, and targeting any stages in cancer progression (Abotaleb *et al.*, 2020). *M. philippinensis*, a close relative species of MC contains an abundance of phenolic compounds, and one that shows effective cytotoxic activity is the phytoconstituent, rottlerin. It was proven that this compound contains cytotoxic activity when tested against 14 human cancer cell lines (Sharma, 2011). Rottlerin is an NF- κ B inhibitor in various cell types. This phytochemical can prevent NF- κ B migration in primary microvascular endothelial cells. Besides this, rottlerin also increases cytoplasmic calcium concentration and stimulates the production of antiproliferative NO thus preventing synthesis, release, and activity of Endothelin (ET)-1, a known

mitogenic factor. Rottlerin is also an antioxidant. Through decreasing oxidative stress and damage, transcription factors for NF- κ B expression are inhibited thus angiogenesis is also prevented (Maioli *et al.*, 2010). Phenolic compounds contain an OH-group in their chemical composition and when interacted

with steroidal (e.g., cardiac glycosides) skeleton may make the phenolic compounds reduce their ability due to sterical hindrance (Weng *et al.*, 2014). This is the reason why crude extract was ineffective in inhibiting the cancer cells due to the interaction of phenolic compounds to cardiac glycosides.

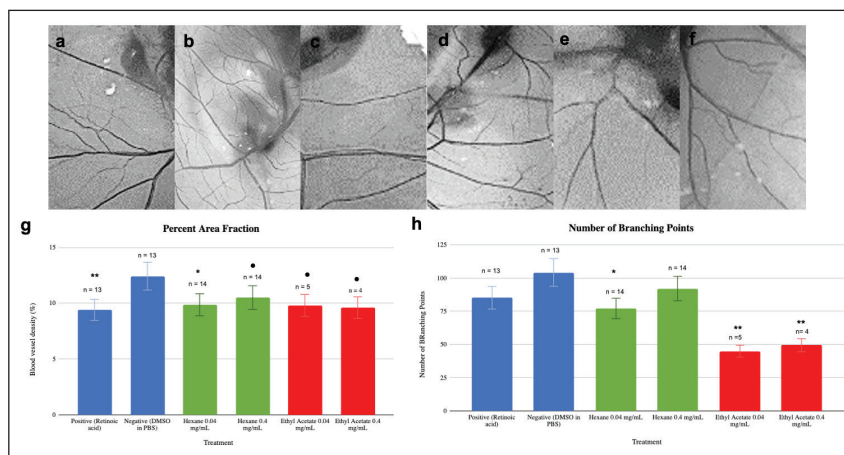


Fig. 3. Images of Egg from control, hexane, and ethyl acetate extract set-ups. Shown were sample images of: (a) positive treatment (b) negative treatment; (c) hexane 0.04 mg/mL treatment; (d) hexane 0.4 mg/mL treatment; (e) ethyl acetate 0.04 mg/mL treatment, (f) ethyl acetate 0.4 mg/mL treatment. (g) represents the percent area fraction in the control, hexane, and ethyl acetate treatments. (h) represents the number of branching points in the control, hexane, and ethyl acetate treatments. ● $p < 0.1$; * $p < 0.05$, significant difference compared to negative control.

CONCLUSION

The anti-cancer and anti-angiogenic activities of the extracts can be attributed to the phytochemicals detected in the study. Among the three extracts, hexane was found to be the most effective in terms of anti-cancer and anti-angiogenesis while maintaining its non-toxic effects. The role of phenolic compounds in the hexane fraction can play a role in this mechanism and further isolation is recommended. Overall, the research proved that *M. cumingii* has the potential to be used for the creation of anti-cancer drugs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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