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Evaluation of Antiangiogenic, Cytotoxic and *In vitro* Antiproliferative Activities of *Alpinia galanga* Leaves Ethanol Extract

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Abstract

Medicinal plants are an important source of natural treatments for a multitude of chronic illnesses like cancer. In this study, ethanol extract from the leaves of *Alpinia galanga* (AGEE) was evaluated for its anticancer potential. Chorioallantoic membranes (CAM), *Tripneustes gratilla* fertility, and MTT assays were conducted to investigate the angiangiogenic, cytotoxic and anti-proliferative activities, respectively. Standard phytochemistry tests were conducted to identify the bioactive compounds present. The CAMs treated with AGEE demonstrated signs of vascularity assault and significantly lowered blood vessel formation, branching point number and branching point density ($p<0.05$). The extracts also exhibited cytotoxicity with a significant reduction in the number of viable sea urchin zygotes as compared to control ($p<0.05$). Finally, AGEE portrayed evidence of antiproliferative activity against HCT-116 with pronounced growth inhibition of 53.01% and IC_{50} of 65.25 µg/ml. A less striking effect in MCF-7 was signified by the minimal inhibitory rate of 12.41% and IC_{50} of 101.4 µg/ml. AGEE showed positive indications for metabolites of flavonoids, sterols and tannins. Total phenolic content (TPC) was quantified at 36.71 gallic acid equivalents (GAE/g). The results suggest the potential of *A. galanga* as a reasonable prospect in the development of natural product-derived anticancer drugs.

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Introduction

In the Philippines, cancer is among the top three leading causes of death, next to heart-related conditions and followed by cerebrovascular diseases (PSA, 2021). According to the data released, cancer has accounted for 467,915 deaths from 2015 to 2020, with the highest mortality incidence from breast and lung cancer patients. The situation is in parallel with the global health statistics, making cancer responsible for 10 million deaths in 2020 (Ferlay *et al.,* 2020). As analyzed, developing countries are more susceptible to this threat (Nelson *et al.,* 2020). Cancer is a genetic disease characterized by the uncontrolled proliferation of abnormal cells. Hanahan and Weinberg (2011) summarized the hallmarks of cancer biology that enable success in the exponential growth of unregulated cells. Included in this enumeration is the induction of blood vessel formation or angiogenesis, cellular senescence or cessation of mitosis, and resistance to programmed cell death or apoptosis.

Advances in oncological research provide remarkable progress in cancer treatment. Emerging technologies in chemotherapeutics, nanomedicine, cancer vaccines, gene therapy, thermal ablation and magnetic hyperthermia, and recent innovations in radiomics and pathomics (Pucci *et al.*, 2019), as well as aesthetic remedies like the fillers, botulinum neurotoxins (BoNTs) and laser therapies (Proietti *et al.*, 2021), are being evaluated and introduced in the clinical trial in the aim of alleviating the burden of cancer. However, despite the novel interventions, challenges continue to arise as the occurrence of drug resistance and unaffordability and inaccessibility to treatments are continuously being reported (Niraula *et al.*, 2014; Ye *et al.*, 2019). Further, deleterious side effects of these treatments ranging from fatigue, insomnia, cognitive impairment (Palesh *et al.*, 2018) to alopecia, hyper/hypopigmentation, onycholysis, vitiligo, xerosis and oedema are experienced (Proietti *et al.,* 2021). With the world's current state of the pandemic, cancer patients treated with cytotoxic chemotherapy are found to be at higher risk of contracting COVID-19 (Lee *et al.*, 2020). Plant-derived compounds are the attractive target of cancer therapeutic agents that promise minimal toxicity. For updates, 62 of the 185 (33%) small molecules in the treatment of the disease are recorded to have originated from natural products (Newman and Cragg, 2020).

Alpinia galanga is a common herb that is found abundantly in the tropics like the Philippines. This herb is locally called *langkuas* and belongs to the Zingiberaceae family. As a botanical relative to ginger, it has multitudes of ethnomedicinal uses in many rural areas, paying much attention to its rhizome for its anti-diabetes, carminative, aphrodisiac, abortifacient, antipyretic and antispasmodic activities (Kaushik *et al.*, 2011). It has traditional benefits against microbial infections, rheumatic pains and dyspepsia (Chouni and Paul, 2018). Recently, the plant has been gaining recognition as a common herb with myriads of positive biological activities. Ethanol extracts from the rhizomes of *A. galanga* are recently reported to have positive pharmacological activities such as antigenotoxic (Nag *et al.*, 2021), neuroprotective (Farkhondeh *et al.*, 2020), ulcerogenic (Johnley *et al.*, 2020) anti-oxidant and antimicrobial (Pillai *et al.*, 2019), antimycobacterial (Alajmi *et al.*, 2018), anticolitic (Baldo and Serrano, 2016) and many more. A recent study of the essential oil from this plant also showed promising immunopotency (Alif *et al.*, 2021). Because of its immune-enhancing effect, natural compounds from *A. galanga,* identified as aceto cavicol acetate (ACA), galangin and 1,8-cineole are now being explored as anti-covid treatment (Utomo *et al.*, 2020).

While the potential of *A. galanga* rhizomes is extensively documented, there is a clear paucity of available literature about its leaves. Jirovets and his colleagues (2003) pioneered the study of essential oils extracted from the leaves, but no work has yet to explore other biological activities. Thus, this study is aimed to elucidate the preliminary anticancer potential of the leaves of *A. galanga* ethanol extract (AGEE) and reveal the active phytochemicals present.

Materials and methods

Plant material

Fresh leaves of *A. galanga* were collected from its natural habitat in Sitio Cawayan, Pandanan, in Caramoan Natural Park (13.4526°N, 123.5643°E) with permission from the Protected Area Management Board (Gratuitous Permit No. R5-119). Plant specimens were authenticated at the Jose Vera Herbarium, University of the Philippines Diliman, Quezon City, Philippines.

Preparation of ethanol extracts

A. galanga leaves were washed thoroughly with running water and air-dried for three days. Leaves were cut into smaller sizes and reduced into a fine powder using an electric blender. Pulverized leaves were soaked in 95% ethanol following a 1:5 ratio (w/v) for 72 hours. Thereafter, the mixture was filtered using Whatman paper No. 42. The solution was evaporated *in vacuo* using a rotary evaporator (IKA-RV10) at 40°C under reduced pressure. Extracts were evaporated to dryness and stored at 4°C prior to further bioassays.

Phytochemistry test

Qualitative analysis

The standard protocol described by Mumtaz *et al.* (2014) was adopted with slight modifications to determine the secondary metabolites: Meyer's test for alkaloids, Liebermann-Burchard Test for sterols and triterpenes, Shibita's reaction test for flavonoids, frothing test for saponins, Fehling's test for glycosides, and ferric chloride test for tannins.

Estimation of total phenolics content (TPC)

Determination of TPC was conducted using Folin-Ciocalteu's method applying the conditions from Chandra *et al.* (2014) with slight modifications. Samples and standard readings were measured using a microplate reader (BIOBASE EL10B). Standard gallic acid of varying concentrations (10, 20, 40, 60, 80 and 100 µg/ml) was prepared in 25 ml volumetric flasks. For the test sample preparation, 10 g of AGEE was dissolved in 5 ml methanol. Standards and AGEE at 200 µl ml were mixed with 60 µl of distilled water and 200 µl of Folin-Ciocalteu's reagent. After 5 minutes, 100 µl of sodium carbonate solution (8% w/v in water) was added and volume was filled to 300 µl with distilled water. The mixture was kept in the dark condition for 30 minutes. The absorbance of the blue color indicating phenolic contents was read spectrophotometrically at 765 nm and was calculated in gallic acid equivalents (GAE/g) of dry plant material using the generated standard curve of gallic acid ($0.0089x + 1.8366$, $R^2=0.9618$). All determinations were performed in triplicate.

Chorioallantoic membrane (CAM) vascularity assay

Antiangiogenicity of the extract was determined using the Chorioallantoic membrane (CAM) assay according to the modified procedure of Raga and colleagues (2013). Fertile mallard duck (*Anas platyrhynchos*) eggs were purchased from a commercial supplier in Ubaliw, Polangui Albay, Philippines. Briefly, three-day-old eggs were incubated at 37°C with constant humidity at the Partido State University Natural Science Laboratory. On the fifth day of incubation, the eggs were candled and inspected for viability. On the seventh day, eggs were cleaned with 70% isopropyl alcohol. A hole was made on each egg by scratching the blunt end with a sterile probe. Each egg was aseptically introduced with 0.2 ml of test concentrations 0.1,1 or 10 µg/ml of AGEE using a sterile syringe. Ethanol was used as a control while a group of eggs remained untouched for environmental control. Thereafter, the inoculated membrane was sealed and the eggs were further incubated. Eggs were monitored constantly to check for embryo survival. Fertile eggs were characterized by a dark spot where blood vessels radiate from. On the 14^{th} day, the eggs were positioned on their lateral side to harvest the CAM. The hard shell was removed, exposing the membrane that protects the developing embryo. The CAM area was visually assessed for vascular damage using a stereomicroscope. The frequency of damage was determined by counting the number of appearances of the most severe damage observed. Any damage on vasculature and obstruction to normal blood flow was considered a positive antiangiogenic effect. The CAMS were photographed

for the assessment of primary blood vessels (PBV), secondary blood vessels (SBV) and tertiary blood vessels (TBV). Branching Point Number (BPN) was also determined. Primary blood vessel thickness was measured using Image J Software. Branching point density was computed using the formula used by Raga *et al.* (2013):

$$Branching\ Point\ Density = \frac{Number\ of\ branch\ point\ in\ a\ blood\ vessel\ segment}{Length\ of\ blood\ vessel\ segment}$$

Sea urchin fertility assay

The sea urchins (*Tripneustes gratilla*) were collected at Atulayan Island, Sagñay Camarines Sur (13.5846°N, 123.5700°E). Test subjects were induced to spawn by injecting 2 ml of 0.5 M KCl solution into the perivisceral cavity. Collected gametes were used for the fertilization by mixing 1 ml of diluted sperm suspension and 1 ml of eggs dispersed in filtered seawater (FSW). Fertilization was noted with the notable appearance of the fertilization membrane. Suspended zygotes or fertilized eggs at 1 ml were pipetted into 24-well plates (Sousa *et al.*, 2009), where it was resuspended with 0.02 ml of AGEE at either 0.01, 0.1 and 1.0 mg/ml concentrations. Dimethyl sulfoxide (DMSO) and FSW were used as controls. After 20 minutes, the egg suspension was collected and fixed in 10% formaldehyde solution. The total number of viable zygotes in each treatment was recorded. The half-maximal inhibition concentration (IC_{50}) was calculated using the Quest Graph™.

3-(4, 5-dimethyl thiazoyl-2-yl)-(2,5-diphenyltetrazolium bromide) (MTT) assay

Cytotoxic activity of AGEE was tested on human colorectal carcinoma (HC-T116) and Michigan Cancer Foundation (MCF-7) cancer cell lines. Briefly, cells were seeded into each well of 96 well microtiter plates at 4 $\times 10^4$ cells/ml. The plates were incubated overnight at 37°C and 5% CO_2. The cells were treated with various concentrations of AGEE (100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78125 µg/ml) and incubated for 72 hours. Doxorubicin was used as the positive control, while DMSO served as a negative control. After incubation, the media were discarded and MTT reagent diluted at 0.5 mg/ml phosphate-buffered saline (PBS) was added and incubated in the same conditions for another 4 hours. The medium was removed and DMSO was added. Optical density (OD) was quantified at 570 nm wavelength (Mosmann, 1983). Percent growth inhibition of treated cancer cells was determined using the formula:

$$\%\ cell\ Inhibition = \frac{Test\ OD}{Non-treated\ OD}\ x\ 100$$

The computed % cell inhibition was plotted against various AGEE concentrations. The half-maximal inhibitory concentration (IC_{50}) was determined according to the equation of the plot (Wong *et al.*, 2014).

Statistical analysis

The data were analyzed using a one-way analysis of variance followed by a post hoc Tukey's test to determine significant difference set at $p \leq 0.05$. The statistical analysis was carried out using SPSS version 26.0 (SPSS Inc., Chicago IL, USA). Data were shown as mean ± standard error of means (SEM).

Results

Phytochemical screening

The phytochemical profile of AGEE was summarized in Table 1.

Table 1. Phytochemical profile of *A. galanga* leaves ethanol extract.

Phytochemical constituent	Results
Sterols	+++
Triterpenes	-
Flavonoids	+++
Alkaloids	++
Saponins	+
Glycosides	+
Tannins	+++
Phenols (TPC)	36.71 GAE/g

(+) Traces, (++), Moderate, (+++), Abundant, (-) Absent. Total Phenolic Contents (TPC), Gallic Acid Equivalents/gram (GAE/g).

This table shows that important secondary metabolites were present in the ethanol extract of *A.*

galanga leaves. Flavonoids, tannins and sterols were qualitatively evaluated to be abundant, while triterpene was not detected. Further, the quantitative profile of phenols was found to be considerably high at 36.71 gallic acid equivalents per gram (GAE/g).

Antiangiogenic activity of A. galanga ethanol extract

The antiangiogenicity of AGEE was tested using CAM model. The evaluation was performed by microscopic observation of vascularity assaults and inhibition of angiogenesis or sprouting of blood vessels. Incidence of mortality was also noted. CAMs treated with 0.1 µg/ml AGEE caused 9% mortality at day 13 of incubation.

This group displayed no noticeable anomalies in the CAM. On the other hand, the appearance of a few ghost vessels was observed from CAMs treated with 1 µg/ml AGEE (Fig. 1). The mortality rate in this group was high at 57%. Group treated with 10 µg/ml AGEE showed characteristics of vascular damage such as the formation of ghost vessels and numerous petechial hemorrhages.

Table 2. Blood vessel formation in different treatments.

Treatment	PBV	SBV	TBV	PBV thickness
Untouched	3.4±0.24^{a}	28.0±1.87^{a}	53.2±3.48^{a}	37.5±3.15^{a}
0.1 µg/ml AGEE	3.2±0.20^{b}	26.8±1.80^{a}	45.8±2.70^{a}	30.7±2.65^{a}
1 µg/ml AGEE	2.2±0.24^{b,c}	12.0±1.54^{b}	18.0±2.30^{b}	20.9±2.03^{b}
10 µg/ml AGEE	1.8±0.20^{c}	4.4±0.67^{b}	5.4±1.20^{b}	17.4±1.43^{b}

Values are expressed as mean±SEM. Values labelled with the same letters are not significantly different ($p \leq 0.05$). PBV- Primary Blood Vessels, SBV-Secondary Blood Vessels; TBV- Tertiary Blood Vessels. AGEE- *A. galanga* Ethanol Extract. (n=7).

Also, the mortality was tallied at 57%. The negative control group treated with ethanol established 100% mortality. The CAM from the untouched group exhibited well-vascularized blood vessels and an extensive junctional complex that gave rise to several contact points (Fig 1A.).

Table 3. Anti-proliferative activity of *A. galanga* leaves ethanol extract.

Concentration (µg/ml)	HCT116	MCF7
	% Inhibition	% Inhibition
100	53.01±4.77	12.41±1.79
50	22.27±5.59	2.12±2.24
25	3.47±3.88	-2.24±7.22
12.5	3.95±2.85	-0.05±4.53
6.25	2.30±3.52	1.70±4.00
IC_{50} (µg/ml)	65.25	101.4

% inhibition is expressed as Mean±SEM. n=3.

Blood vessels were counted to assess the branching complexity (Table 2). AGEE-treated CAMs revealed a significant reduction in the formation of PBV, SBV and TBV compared to the untouched group ($p<0.05$) with the exception of the lowest dose presenting normally branched blood vessels. It is noteworthy that AGEE at the highest dose posed the most active angio-suppression, as shown by the declined number of blood vessels and reduced PBV thickness. The data also correlates with the branching point analysis (Fig. 2). AGEE at 10 µg/ml illustrated an intense ability to decrease the number of branching points and density compared to all treatments ($p<0.05$). No numerical data were recorded for the ethanol-treated because of early records of mortality observed prior to termination.

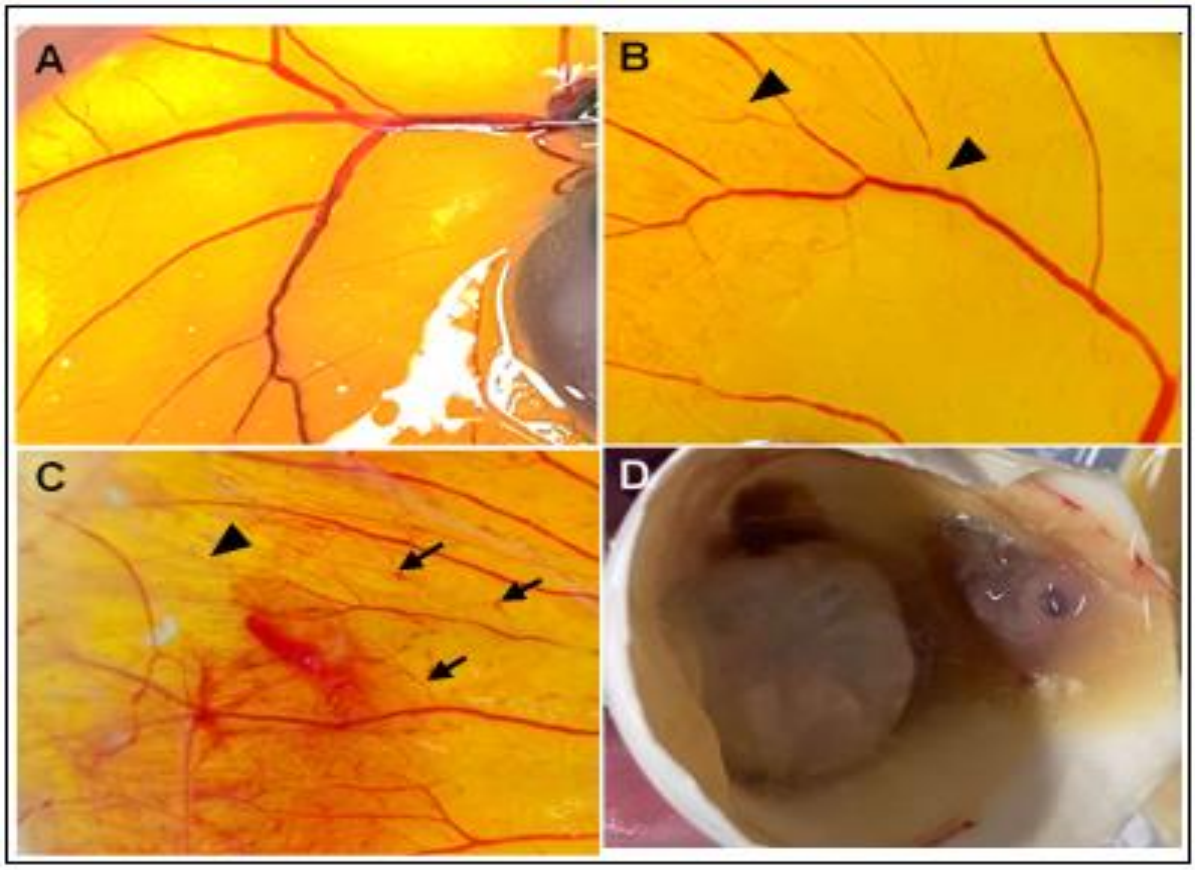

Fig. 1. CAM angiogenesis from different treatments: (A) Untouched, (B) 1 µg/ml AGEE, (C) 10 µg/ml, (D) ethanol. Arrowheads indicate ghost vessels and arrows indicate petechial hemorrhages.

Cytotoxic activity of A. galanga ethanol extract

The cytotoxicity of AGEE was illustrated by the total number of fertilized eggs (Fig. 3). The highest number of viable cells were observed in the FSW-re-suspended wells. On the other hand, a concentration-dependent effect was observed in the survival of AGEE-treated zygotes. A downward trend in the number of eggs was displayed by AGEE at 0.1 mg/ml ($p<0.05$). The highest dose of AGEE showed a highly significant difference in the number of viable cells compared to FSW and DMSO ($p<0.001$). At the lowest concentration, AGEE did not cause a significant effect in lowering the number of viable cells compared to FSW and DMSO. IC_{50} value was recorded at 0.097 mg/ml.

In vitro anti-proliferative activity of A. galanga ethanol extract

The activity of AGEE in the inhibition of cell growth was investigated against Michigan Cancer Foundation-7 (MCF-7), a breast cancer cell line and Human Colorectal Carcinoma (HCT-116), a colon cancer cell line, using MTT assay. Percent inhibition is presented in Table 3. AGEE demonstrated anti-proliferative activity at 100 µg/ml in HCT-116 with growth inhibition greater than 50% and an IC_{50} value of 65.25 µg/ml. However, the plant material showed a weak inhibitory effect against MCF-7 cell proliferation in all concentrations. The most active dose of 100 µg/ml caused minimal inhibitory effect at 12.41% with IC_{50} of more than 100 µg/ml. Doxorubicin was shown to be the best inhibitor with IC_{50} of 0.06 µg/ml for HCT-116 and 0.57 µg/ml for MCF-7.

Fig. 4 shows the photomicrographic images of HCT-116 and MCF-7 cell lines. The cell pictures reveal the effects of AGEE in cellular morphology. In HCT-116, intracellular junctions among cells appeared to lose adherence, compared to untreated cells displaying compact monolayer of actively proliferating cells, characterized by distinct nuclei and unabridged cellular membrane.

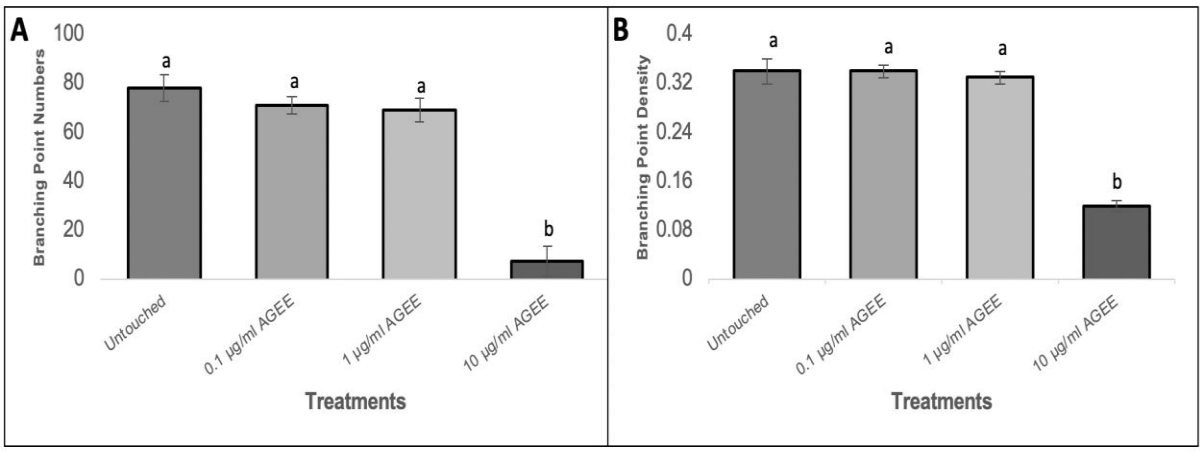

Fig. 2. Analysis of Branching point Number (2A) and Branching Point Density (2B), Means followed by the same letters are not significantly different (p≤0.05). n=7.

Additionally, cells treated with 100 µg/ml AGEE showed membrane blebbing, suggested by small protrusions of the membrane and apoptotic bodies (Fig. 4B). On the other hand, no apparent signs of cell morphology alterations were observed in AGEE-treated MCF-7 cells. Only cells treated with positive control doxorubicin showed apoptotic-like indications (Fig. 4F). Furthermore, cytotoxicity was confirmed by the ability of the extract concentration to inhibit 50% growth of treated cells (IC_{50}). IC_{50} values are computed at 65.25 µg/ml for HCT-116 and 101.4 µg/ml for MCF-7.

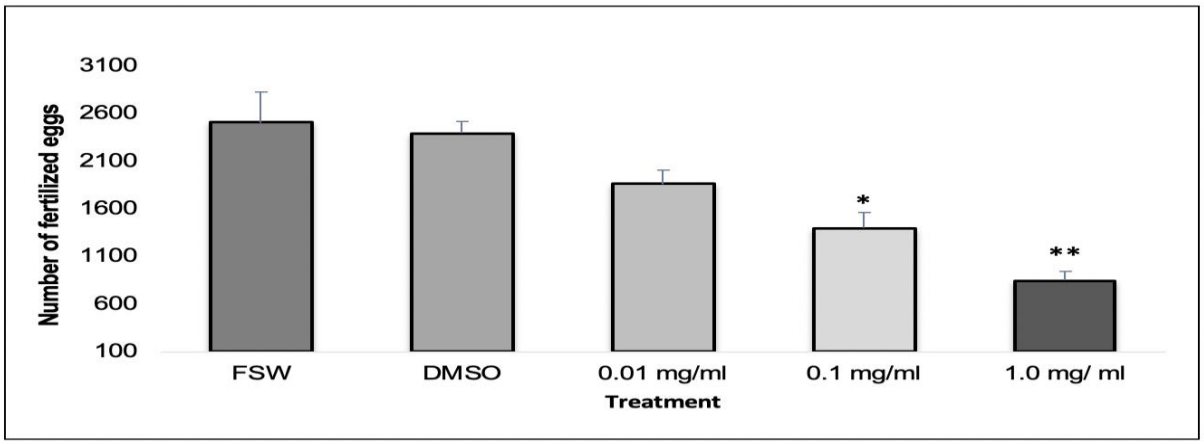

Fig. 3. Number of observed viable zygotes after exposure to different doses of AGEE.
*p≤0.05, ** p≤0.001. FSW- filtered sea water, DMSO-dimethyl sulfoxide.

Discussion

A. galanga leaves ethanol extract demonstrated advantageous effects on the different assays performed, manifesting an overall anticancer pharmacological potential. The extract has shown the capacity to suppress the formation of new blood vessels in the developing duck eggs. Also, it has illustrated efficacy to block successful cellular division as indicated by the decreased number of viable zygotes of sea urchin. Lastly, AGEE presented a noteworthy ability to inhibit the proliferation of cancer cell lines, with a prominent effect on colon cancer and a weaker anti-proliferative effect on breast cancer. Abundant levels of beneficial phytochemical ingredients are detected.

The phytochemical composition of AGEE revealed dominant levels of flavonoids, tannins and sterols.

Total phenolic contents were estimated at 36.71 GAE/g. Numerous studies confirm the presence of these secondary metabolites in the rhizomes and seeds of *A. galanga* (Rao *et al.,* 2011; Bian *et al.*, 2014; Malik *et al.*, 2016; Tungmunnithum *et al.*, 2020). However, data is scarce on the phytochemical profile of the plant's leaves. In a study communicated by Rani and associates (2016), *A. galanga* leaves showed positive results for phenols, but authors failed to present quantification of the contents.

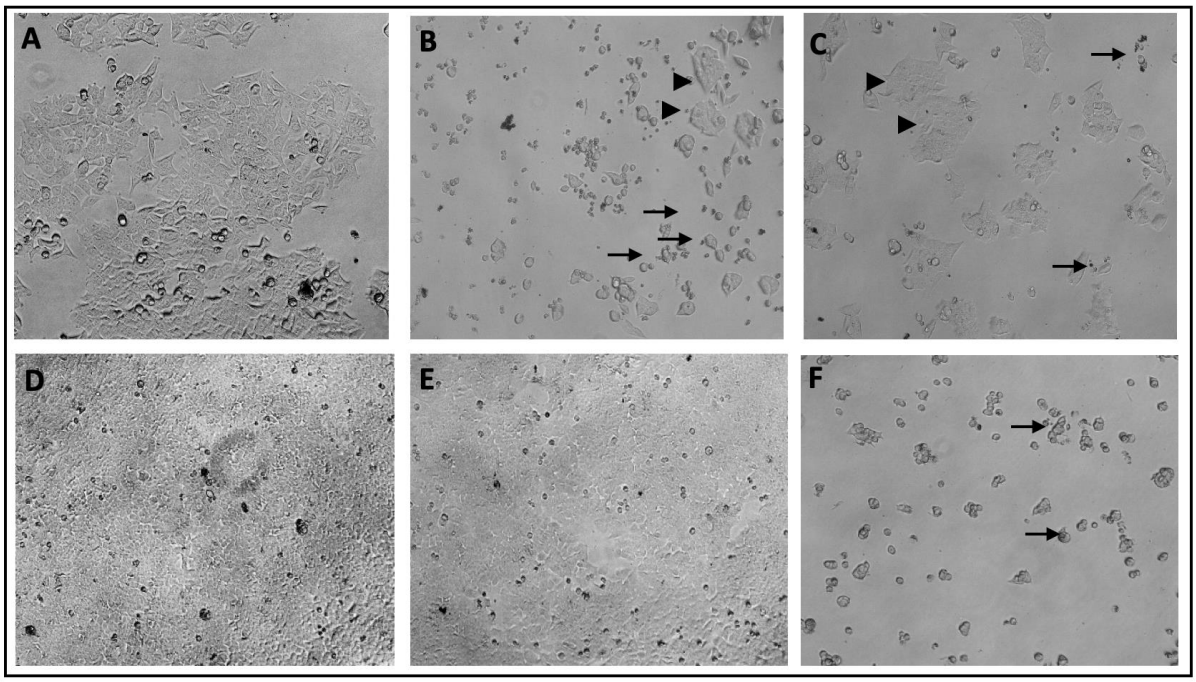

Fig. 4. Morphological changes in HCT-116 (A-C) and MCF-7 (D-F) cancer cell lines treated with different treatments: (A and D) DMSO (B and E) 100 µg/ml AGEE, (C and F) Doxorubicin. Arrowheads indicate membrane blebbing and arrows indicate apoptotic bodies.

Inhibition of angiogenesis is beneficial for the prevention of neoplastic and tumor growth, thus the growing interest to discover novel inhibitors of blood vessel formation from natural products. Vascular damage and blockage in blood flows are positive indications of anti-angiogenic activity (Raga *et al.,* 2013). Most active angio-suppression was observed in AGEE at a high concentration of 10 µg/ml, as proven by photomicrographs showing reduced and assaulted microvasculatures in the CAM. Ghost vessels and petechial hemorrhages are signs that capillaries are either devoid or has increased blood flow (Canoy and Bitacura, 2018), respectively. Of note, the deteriorated vascularization is also associated with the lessening in branching point number and density. These impacts can be linked with the presence of phytochemicals. A high level of flavonoids was revealed in this study. This important secondary metabolite inhibits the generation of fresh blood vessels by regulating specific signaling pathways. In effect, there is a controlled expression of angiogenic factors like vascular endothelial growth factors (VEGF), matrix metalloproteinases (MMPs) and epidermal growth factor receptor (EGFR) (Tsakiroglou *et al.*, 2019; Subbaraj *et al.*, 2021) that blocks angiogenesis. Modulating the basic step of angiogenesis is important because unregulated protrusion of blood vessels leads to pathological conditions like cancer. Additionally, alkaloids that were tested present in high levels target different cellular mechanism that also inhibits angiogenesis (Zhao *et al.*, 2014). Ultimately, phenols expressed in gallic acid equivalents are established anti-oxidants as it blocks reactive oxygen species (ROS) from initiating the process of angiogenesis (Shafaei *et al.*, 2014).

For many years, sea urchin has been utilized as an effective prototype in analyzing pathways and

mechanisms of compounds against cell survival and death (Bernardo and Carlo, 2017). In this study, FSW and DMSO treated groups showed the sustained number of fertilized eggs portraying complete inactivity on cytotoxicity. These findings agree with the report of Sciarrino and Matranga (1995), who mentioned that DMSO does not affect the development of sea urchin embryos development. AGEE at 1.0 mg/ml showed a highly significant decrease in the viable zygotes compared to the control. These positive actions are attributable to the elevated amount of sterols that are present in the extracts. Plant sterols, often referred to as phytosterols, are made up of complex structures like cholesterols, campesterol, 24-methyl sterol and many more (Dufourc, 2008). According to Chen (1984) high amounts of cholesterol inhibit cellular differentiation, a possible mechanism behind the results of this assay.

In this study, substantial pieces of evidence show that AGEE at 100 µg/ml has anti-proliferative ability as indicated by a high rate of growth inhibition in HCT-116. These findings agree with the work of Kang and his colleagues (2009), who reported that Xanthorrhizol, isolated from *Curcuma xanthorrhiza,* can induce apoptosis, and thus initiate cell cycle arrest in the human colon cancer cell line. This plant species is botanically related to *A. galanga* and xanthorrhizol's cytotoxicity could be boosted by its phenol group (Oon *et al.*, 2015), which is present in the extract. Apoptosis induction is shown by the abnormalities in cells, such as membrane blebbing and the formation of apoptotic bodies. Lower IC_{50} profile corresponds to the high toxicity of AGEE against cancerous cell lines. Although AGEE presented the potential to block cell growth, the IC_{50} is not as good as the result of the doxorubicin (0.06 µg/ml), the positive control. Bugayong and Jacinto (2017) predicted that as crude extracts undergo the purification process, more acceptable IC_{50} will be generated. Meanwhile, AGEE was shown to be less sensitive to MCF-7 as supported by IC_{50} greater than 100 µg/ml and preserved cancer cell morphology.

Conclusion

To the author's knowledge, this is likely the first report to describe the anticancer activity and phytochemical profile of *A. galanga* leaves. The plant exhibits potent antiangiogenic, cytotoxic and anti-proliferative activities. Ergo, it can be tapped as an efficient candidate for the natural product-derived anticancer agent. As well, the authors recommend the use of non-cancerous cells in future viability assays to establish the safety of the extract on normal cells.

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