RESEARCH ARTICLE

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Assessment of Cytotoxicity, Impact on Cell Migration and Apoptotic Modulation of Acteoside and Plantamajoside on Human Breast Adenocarcinoma (MCF-7)

Kyle Andrei Cabatit[1], Lara Justine Carandang[1], Dianne Joyce Saragpon[1], Khayria Minalang[2], John Paulin[1], Mark Kevin Devanadera[1,2]*, Minerva Daya[1,2]

Abstract

Objective: To evaluate the anticancer potential of Acteoside and Plantamajoside using MCF-7 cancer cell line. **Methods:** In this study, the half-maximal inhibitory concentration (IC_{50}) values of Acteoside and Plantamajoside were investigated using the Sulforhodamine B (SRB) assay. The IC_{50} values of both compounds were utilized in the clonogenic and scratch wound assays to assess cell survival and migration, respectively. The cells with treatments were also analyzed using the Caspase 3/7 assay to determine their capability to induce cell apoptosis. Network Pharmacology was used to evaluate their probable protein targets and pharmacological mechanisms. **Results:** Acteoside and Plantamajoside exhibited cytotoxic activity against the MCF-7 cancer cell line *in vitro,* with IC_{50} values of 134.83 µg/mL and 225.10 µg/mL, respectively. This was further supported by clonogenic assay, which showed a difference in colony formation following treatment with ACT and PMS, compared to the negative control. Specifically, ACT resulted in the formation of only 39.7% of colonies, whereas PMS formed 51.12% of colonies, indicating that these compounds impaired the cells' ability to proliferate and form colonies. Moreover, these compounds were also able to inhibit cell migration as a reduction in the migration area of the MCF-7 cell line was observed. An increase in the percentage of apoptotic cells was also noted post-treatment, which correlated with elevated Caspase 3/7 activity, indicating that these compounds may effectively induce cellular apoptosis. *In silico* predictions demonstrated that the compounds' anticancer effect may be attributed to their interactions with TLR, PI3K, and STAT—all are implicated in the PD-1 checkpoint pathway and *PD-L1* expression in cancer. **Conclusion:** Both Acteoside and Plantamajoside have demonstrated promising anticancer properties by inhibiting the growth and metastasis of MCF-7 cancer cell line. These compounds induce apoptosis, modulate the PD-1 checkpoint pathway, and influence *PD-L1* expression, which may indicate possible molecular mechanisms for their anticancer effects.

Keywords: Plantago- Network Pharmacology- Pure Compounds- Anticancer

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Introduction

Globally, cancer continues to be a major cause of death, accounting for approximately 10 million deaths in 2020 alone, according to the information provided by the World Health Organization's (WHO) International Agency for Research on Cancer (IARC). Among the various types, breast cancer emerged as the most prevalent and aggressive, with 2.26 million reported cases in the same year. In the Philippines, breast cancer remains a significant health challenge due to various factors, which include socioeconomic disparities. Thus, the search for a cost-effective and safe treatment for breast cancer has been one of the concerns of the healthcare community.

Synthetic drugs such as doxycycline (DOX) may have been readily available as chemotherapeutic treatment. However, the prolonged use and high concentrations of this drug may lead to toxic effects [1]. Therefore, the use of the compounds that can be found in the *Plantago* genus have been considered as a substitute for these synthetic drugs for potential anticancer activity. The *Plantago* genus, a member of the Plantaginaceae family, comprises roughly of 275 species that can be encountered across the globe. Recent study has determined that the methanolic extract of *Plantago* species possess significant cytotoxic activity against cancer cell lines, one of which is the MCF-7 breast cancer [2]. The *Plantago* genus contains bioactive compounds such as iridoid glycosides and phenylethanoid glycosides, which have shown potential anticancer properties. These compounds play

*[1]Department of Biochemistry, Faculty of Pharmacy, University of Santo Tomas, Philippines. [2]Research Center for Natural and Applied Sciences, University of Santo Tomas, Philippines. *For Correspondence: mpdevanadera@ust.edu.ph*

roles in regulating cancer cell invasion, metastasis, and proliferation [3]. Acteoside (ACT) and Plantamajoside (PMS) are two notable phenylethanoid glycoside compounds that are commonly found in the *Plantago* genus [4]. ACT has demonstrated superior inhibition of cancer cell proliferation compared to other compounds like echinacoside and calceolariosides [5]. Moreover, ACT exhibits antitumor activity by promoting programmed cell death, preventing cancer spread, and suppressing growth through various pathways [6]. PMS, which shares structural similarities with ACT, exhibits promising effects on cancer cell behavior as it was able to inhibit MMP2 and MMP9 activity, reducing cancer cell growth, movement, and invasion [7]. These findings position these two compounds as key candidates for further exploration in anticancer therapeutic applications.

This study provides relevant information about the bioactivity of the plant metabolites found in the *Plantago* genus - ACT and PMS. In this research, we assessed the anticancer property of the compounds by evaluating their effect on cell viability, proliferation, and migration. The capability to induce apoptosis was observed through the activation of the Caspase 3/7. The interaction of the compounds with protein targets as well as its pharmacological mechanism associated with anticancer activity was determined *in silico.*

Materials and Methods

Sample Procurement and Preparation

ACT and PMS were procured from Naturewill Biotechnology Co., Ltd. (Sichuan,China), ≥98 % pure, dissolved in 0.5% dimethylsulfoxide (DMSO).

Cell Culture

The MCF-7 cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured and grown in Dulbecco's Modified Eagle Medium (Gibco), which was supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/mL streptomycin (Life Technologies, Inc.). The culture was maintained at a temperature of 37°C with a CO_2 concentration of 5%.

Cell Viability Assay

Cell line (1 x 10^5 cells/ml) in the exponential growth phase was treated with 0.25% (v/v) trypsin, seeded in triplicate into 96-well flat bottom microtiter plates, and incubated for 24 hours at 37°C in a humidified incubator with 5% CO_2 gas to allow attachment. The cells were treated with various concentrations of ACT and PMS (50 µg/mL, 100 µg/mL, 200 µg/mL, and 400 µg/mL), along with a positive control (DOX at 50 µg/mL, 100 µg/mL, 200 µg/mL, and 400 µg/mL) and a negative control consisting of media with 0.5% DMSO. After 24 hours of incubation, the culture media containing the treatments was removed, and 10% (w/v) ice-cold trichloroacetic acid (TCA) was added. The plates were incubated in the dark at a temperature of 4°C for 60 minutes. It was then stained using a solution of 0.04% (w/v) SRB in 1% (v/v) acetic acid for 60 minutes, followed by washing with 1% (v/v) acetic acid. Following air drying of the plates, the protein-bound dye was solubilized with 10 mM Tris base (pH 10.5) over a 60-minute period to ensure complete dissolution and optimal conditions for subsequent analysis. The measurement of absorbance at 540 nm was measured in triplicates using a microplate reader (Multiskan Go, ThermoFisher Scientific). The SRB assay was performed in three independent trials.

Cell Proliferation Assay

A total of 1,000 cells were seeded in a 6-well plate and were subsequently treated with IC_{50} of ACT and PMS. Following a period of 5 days, the resulting colonies were washed once with phosphate-buffered saline (PBS) and then subjected to fixation with 4% formaldehyde in PBS. The fixed colonies were incubated at room temperature for approximately 20 minutes. The colonies were washed with PBS and subsequently subjected to crystal violet staining. After a 30-minute incubation, the stain was removed by washing with distilled water, and the plate was subsequently left to air dry. The cells were then counted and analyzed through Image J.

Cell Migration Assay

The cells were grown in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a 6-well plate. The cells were allowed to grow in this culture setup until reaching a confluency of greater than 90%. Using a sterile white pipette tip, the cell monolayers were scratched; after that, they were washed several times in PBS to get rid of the cell debris [8]. The IC_{50} of ACT and PMS were added to the medium in which the cells were cultured. Cell migration was assessed at three distinct time points: 0, 24, and 48 hours post-treatment. At each time point, microscopic analysis and brightfield images of the wound region were taken using the EVOS fluorescence microscope. The migration area was measured using ImageJ software.

Caspase 3/7 Assay

Cellular apoptosis was investigated using the CellEvent™ Caspase-3/7 Green Assay Kit (Invitrogen, Thermo Fisher Scientific). MCF-7 cells (1 x 10^5) were seeded in a 6-well plate and were treated with the IC_{50} of ACT and PMS. After 24 hours, cells were harvested and were stained with CellEvent Caspase-3/7 Green Detection Reagent and were incubated for 30 mins in a 5% CO_2 humidified atmosphere at 37°C. It was then followed by the addition of the SYTOX™ AADvanced™ Dead Cell Stain in DMSO. Caspase 3/7 analysis was performed using CytoFLEX Flow Cytometer (Beckman Coulter, Inc.,USA), with duplicates per experiment and a flow rate of 10,000 events per second.

Network Pharmacology: Target Prediction, Protein-Protein Interaction (PPI) Network Construction, and KEGG pathway analysis

The canonical smiles of ACT and PMS were obtained from PubChem and were used to determine the gene targets of ACT and PMS from Super-PRED (prediction.

charite.de). Differentially expressed genes from breast cancer (BRCA) and normal breast were identified from Gene Expression Profiling Interactive Analysis (GEPIA2, gepia2.cancer-pku.cn). Overexpressed genes were selected using LIMMA and with a cutoff of |log2 FC| > 1 and $p < 0.05$. SynGO (www.syngoportal.org/convert) was used to standardize gene nomenclatures into their official gene symbols as determined by the HUGO Gene Nomenclature Committee. Venny 2.1 (bioinfogp.cnb.csic.es/tools/venny) was then used to match gene targets between the plant metabolites and BRCA.

Protein-protein interaction network between the matched protein-coding genes was then created through the STRING database (string-db.org). Significant proteins are selected based on the following criteria: (1) with more than one interaction, (2) interaction score of more than 0.40, (3) FDR stringency of 0.05 limited to "*Homo sapiens*" species. Cytoscape 3.10.1 with CytoHubba plug-in was then used for topological analysis using degree ranking to determine the top 5 gene targets of ACT and PMS. Afterwards, the top 5 targets were imported into ShinyGo 0.77 (http://bioinformatics.sdstate.edu/go77/) for KEGG analysis and investigation of the pathways and pharmacological mechanisms of ACT and PMS in cancer.

Data Processing and Statistical Analysis

The quantitative data was expressed as mean ± SEM. For the statistical analysis, GraphPad Prism 10 software (GraphPad Software, Inc, San Diego, CA, USA) was used. Two-way ANOVA was used to establish the significance for the comparison of the nominal data. A p-value of 0.05 or less is deemed statistically significant.

Results

ACT and PMS can induce cytotoxic activity against MCF-7 cells

Based on the results obtained, both the plant compounds were able to exhibit cytotoxic activity against the cancer cell line as it was able to decrease the cell viability of the MCF-7 as presented in Figure 1. In the experiment conducted, four different concentrations (50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL) of each of the compounds were tested, and results showed that its cytotoxic effects are concentration dependent as seen in Figure 2. Moreover, ACT has relatively shown a consistent toxicity effect as the concentration increases whereas PMS exhibited weak toxicity at the lowest concentration tested, but it was able to perform comparably to ACT in its treatment at 400 µg/mL ($p > 0.05$). Furthermore, the IC_{50} of ACT and PMS were also investigated using the SRB assay. The IC_{50} was observed at 134.83 µg/mL for ACT and 225.10 µg/mL for PMS. The positive control, DOX, has illustrated a greater IC_{50} value with 343.42 µg/mL.

ACT and PMS reduce MCF-7 colony formation

The colony-forming potential of cells treated with DOX, ACT, and PMS was evaluated using ImageJ software. Compared to the negative control, which showed 100% colony formation, treatments with DOX, ACT, and PMS significantly reduced colony formation as seen in Figure 3, which can also be supported by the percentage colonies formed presented in Figure 4. The administration of DOX treatment led to a significant reduction in the formation of colonies, with only 35.56% of colonies forming in comparison to the negative control showing that it demonstrated the most potent inhibitory effect. Similarly, ACT demonstrated a comparable inhibitory effect, resulting only in 39.7% colony formation. In contrast, PMS treatment showed a less pronounced inhibitory effect, with 51.12% colony formation relative to the negative control.

ACT and PMS inhibit cell migration

The microscopic evaluation of the scratch-wound assay is presented in Figure 5 and the percentage of migration is shown in Supplementary Figure 1. Firstly, the negative control demonstrated a rapid migration, with a migration area of 44.02% at 24 hours, and 53.99% at 48 hours, serving as the standard by which the other treatments were compared. Furthermore, cells treated with the IC_{50} of DOX exhibited an excessive reduction in migration area, with 6.8% at 24 hours, and -16.50% at 48 hours. This decrease in migration area may be attributed to

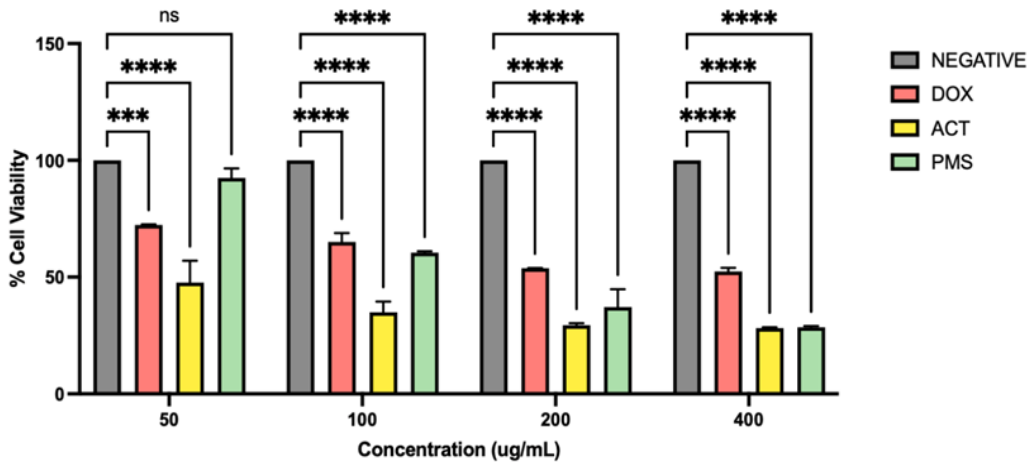

Figure 1. MCF-7 Cell Viability after Treatment with Varying Concentrations of DOX, ACT, and PMS. Results are expressed as mean ± SEM (bars), (Note: *p<0.05 significant differences against the negative control).

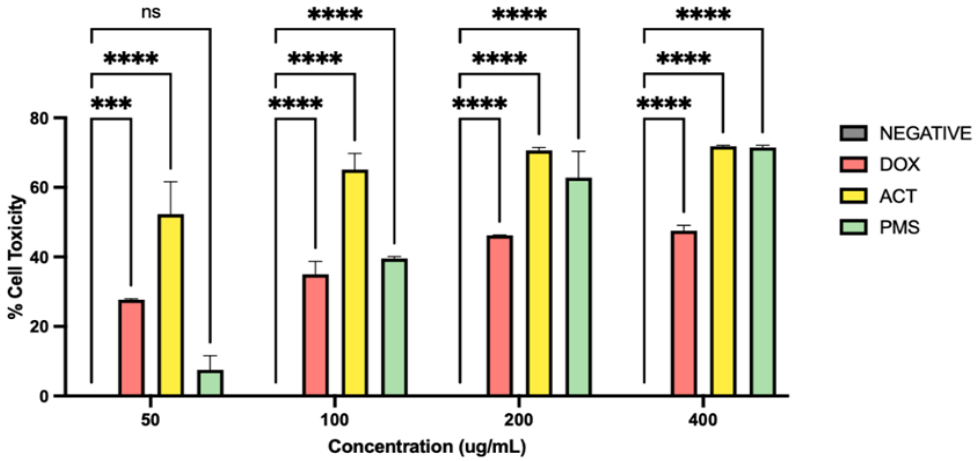

Figure 2. DOX, ACT, and PMS Cell Toxicity against MCF-7 at Different Concentrations. Results are expressed as mean ± SEM (bars), (Note: *p<0.05 significant differences against the negative control).

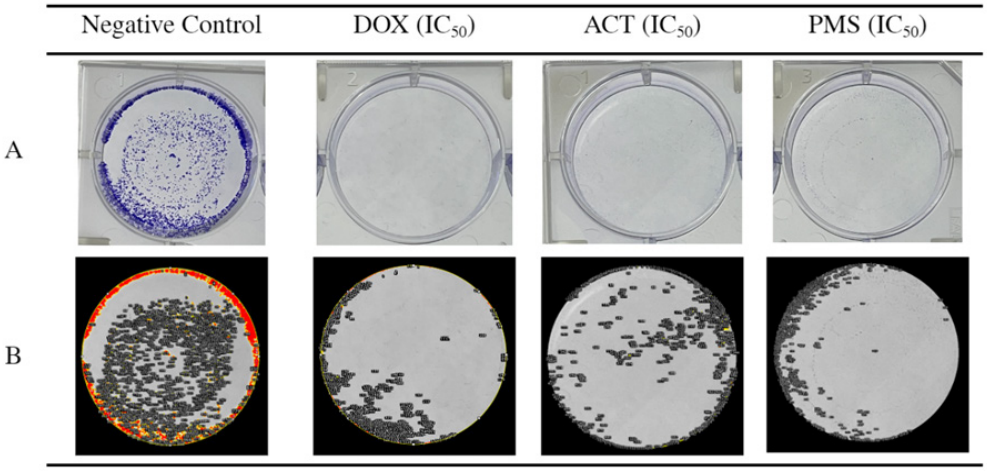

Figure 3. (A) Images of the MCF-7 colonies formed after treatment of DOX, ACT, and PMS. (B) Image J analysis of the colonies formed.

the cytotoxic nature of DOX, acting as a positive control by inducing cell death and detachment over time, resulting in a minor expansion of the wound area rather than closure. In contrast, cells treated with the IC_{50} of ACT showed decreased migration area, with 14.38% at 24 hours, and 25.36% at 48 hours. Similarly, cells subjected to IC_{50} of PMS exhibited a reduction in the migration area, with 12.36% at 24 hours, and 15.97% at 48 hours.

ACT and PMS modulate apoptosis by stimulating Caspase 3/7 activity

The study conducted focuses on the ability of these compounds to modulate apoptosis through the activation of Caspase 3/7. Supplementary Figure 2 and Table 1

Table 1. Percentage of Apoptotic Cells in MCF-7 Cells Treated with IC_{50} of DOX, ACT, and PMS.

	Apoptotic Cells (%)
Negative Control	5.01%
DOX IC_{50}	97.39%
ACT IC_{50}	26.94%
PMS IC_{50}	11.51%

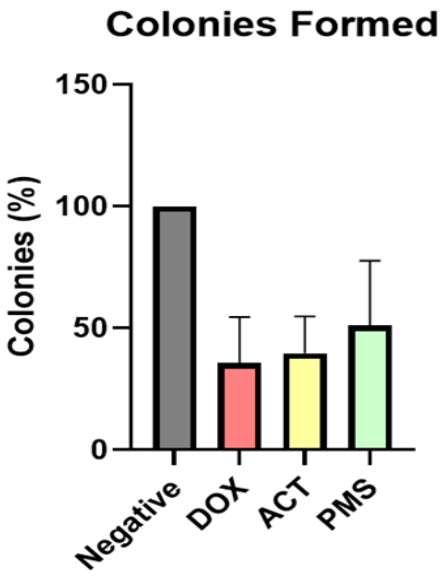

Figure 4. Percentage of Colonies Formed after IC_{50} Treatment of DOX, ACT, and PMS. Results are Expressed as mean ± SEM (bars).

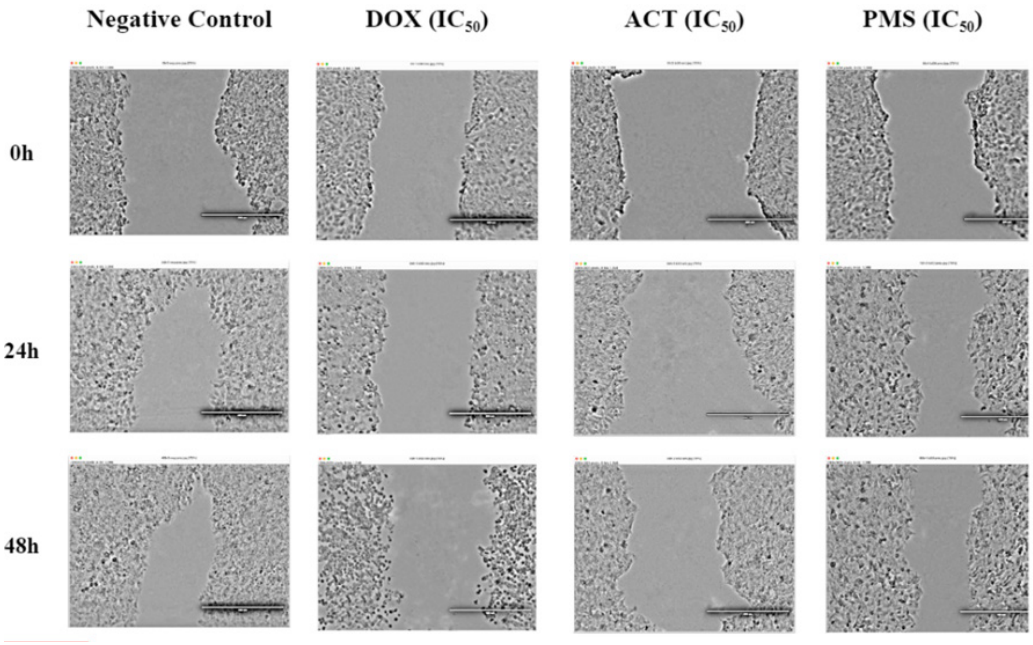

Figure 5. Microscopic Evaluation of Wound Healing Assays in MCF-7 Cells Treated with IC_{50} of DOX, ACT, and PMS: Time-course Analysis at 0, 24, and 48 Hours.

from the study's results demonstrate that treatment of the MCF-7 cells with DOX, ACT, and PMS causes an increase in the number of apoptotic cells. ACT-treated cells rise to 26.94% and PMS-treated cells to 11.51% from the 5.01% apoptotic cells of the negative control. A significant difference was observed with DOX as most of the cells (97.39%) had already reached the apoptotic state. Furthermore, these percentages were obtained from the cells going through late-stage apoptosis, which are in the upper right quadrant, and the early apoptotic cells, which are positioned in the bottom right quadrant.

Network Pharmacology

The target prediction results, as shown in Supplementary Figure 3, indicate that ACT targets 85 genes while PMS targets 102 genes. A total of 28 gene targets have been linked to the anticancer effect of these plant compounds, based on gene intersections between the overexpressed genes in BRCA and these plant compounds. Supplementary Figure 4 presents the specific gene targets associated with ACT and PMS.

A network of connections between proteins was created and is shown in Supplementary Figure 5 The top 5 gene targets were identified by topological analysis using degree ranking: Heat Shock Protein 90 Alpha Family Class A Member 1 (HSP90AA1), which is a target of PMS exclusively; Phosphoinositide-3-Kinase Regulatory Subunit 1 (PI3KR1); Signal Transducer and Activator of Transcription 1 (STAT1); Cyclin-dependent kinase 1 (CDK1); Toll-like receptor 4 (TLR4), the remaining four are targets of both ACT and PMS. Moreover, the KEGG pathway database's enrichment analysis presented in Supplementary Figure 6 has demonstrated that ACT and PMS are particularly linked to the Programmed Death-1 (PD-1) checkpoint pathway and *PD-L1* expression in cancer. It specifically interacts in PI3K/Akt, JAK/STAT, and TLR signaling pathway.

Discussion

Plantago extracts demonstrate anticancer potential, with methanolic extract exhibiting cytotoxic effect to CAL51 cells [9] and alcohol/acetone extracts inhibiting HCT-116 and SW-480 cells [10], which may be linked to the presence of phenylethanoid glycosides such as ACT and PMS. ACT demonstrates antimetastatic, antioxidant, and chemopreventive properties. It reduces metastasis in melanoma cells [11], scavenges free radicals to protect against CCl_4-induced liver damage [12], and prevents DEN-induced liver cancer by reducing hepatocyte nodules and reversing toxicity [13]. Furthermore, it exhibited increased toxicity when tested on various cancers like osteosarcoma and mouse cancer cell lines - C5N and A5 [14]. ACT can also enhance ROS production in tumor cell lines which restricts the ability of the tumor cells to activate cytoprotective pathways. Additionally, ACT's ER-mediated strong antiestrogenic action in the ERa and ERß systems may reduce cell viability [15]. On the other hand, PMS exhibits antioxidant, anti-inflammatory, and antiglycation activities. These properties contribute to its protective effects against oxidative stress, inflammation, and conditions associated with advanced glycation end products [16]. Moreover, it was able to decrease the cell viability of human and mouse breast cancer cells (MDA-MB-231 and 4T1 cell lines) in a dose- and time-dependent manner [7]. The mechanism of PMS involves its interaction with MMPs, which are prognostic markers for breast cancer. In fact, the study demonstrates that PMS is able to reduce the expression of MMP-2 and MMP-9, which are key enzymes involved in cancer progression [7].

Toxicity assessments reveal that PMS does not have any harmful effects on normal melanocytes [17]. In a separate study, ACT was also found to have no toxic effects on normal human diploid fibroblasts [14]. In contrast, ACT demonstrates potent cytotoxic effects

against various cancer cells, primarily due to its catechol moieties and rhamnose group, which are crucial for its activity. It induces G0/G1 cell cycle arrest, inhibits HL-60 leukemia cell proliferation, and promotes differentiation into monocytes [18]. On the other hand, study have also found that PMS enhances the cytotoxicity of metformin in liver cancer cells by amplifying its effects on proliferation arrest, migration, invasion, apoptosis, and autophagy. The combination of PMS and metformin suppresses tumor growth through Akt/GSK3β signaling, highlighting its potential to improve liver cancer treatment [19]. These results complement the study's findings, which show that PMS and ACT are, in fact, cytotoxic to cancer cells. In general, the results of the study correspond to a recent study since the IC_{50} of ACT and PMS from their study, which was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [20], is comparable to the IC_{50} that was obtained using the SRB assay in the experiment conducted. The SRB assay was utilized in the study as it provides better sensitivity, reproducibility, and a stable end-point, making it a preferred option for cytotoxicity testing [21]. This method uses SRB, a dye that binds to cellular proteins, with the amount of dye taken up correlating with cell number.

Cell proliferation is a crucial component of many biological processes, such as tissue growth, wound healing, and cancer progression [22]. In the clonogenic assay, the number and size of colonies formed provided significant information about the proliferation capacity and survival of cells under various experimental conditions. In the study conducted, results suggest that DOX exhibits the most potent inhibitory effect based on the cell viability results. This finding is consistent with a study where DOX treated cells formed significantly reduced colonies compared to the negative control [23]. ACT has shown the ability to effectively inhibit tumor growth in various cell lines, including BEL7404, HLF, and JHH-7, which are human hepatocellular carcinoma cell lines [24]. On the other hand, PMS exhibited the highest number of colonies generated among the treatments. PMS still demonstrated an inhibitory effect; however, it is less pronounced than DOX and ACT. Consequently, the anti-proliferative activity of PMS shown can be supported by a study which showed that PMS effectively inhibited the migration and invasion of HepG2 cells induced by $CoCl_2$ [25]. The study found that the vitality of HepG2 cells reduced when the concentrations of $CoCl_2$, a hypoxia-mimicking agent, and/or PMS increased; This suggests that PMS has an impact on the proliferative activity of cancer cells [25].

A crucial process known as metastasis, which is facilitated by cell migration, is what causes cancer to propagate and progress. In this study, the migration of MCF-7 cancer cells was observed through the scratch-wound assay following treatment with compounds DOX, ACT, and PMS. Observations were made at 0, 24, and 48 hours to analyze the changes in wound healing and cell migration, with subsequent evaluation using ImageJ software. When the migration area is higher, it indicates that the wound is healing faster, implying faster cell migration. Conversely, a lower migration area (e.g., compared to the negative control), suggests slower wound healing, indicative of slower cell migration. The findings of the study suggest that ACT effectively reduced the migration of MCF-7 cancer cells compared to the negative control. This coincides with a research investigation which states that ACT inhibits the migration of breast cancer cells [26]. In a similar manner, cells treated with PMS also showed a decrease in the migration area at both 24 and 48 hours. This can be supported by the fact that PMS suppresses malignant melanoma cell migration and invasion in a dose-dependent way [17]. While PMS also demonstrated an inhibitory effect on cell migration compared to the negative control, it appeared to have a lesser effect than ACT. Overall, the results of the SRB and clonogenic assays are consistent with the findings from the scratch-wound experiment. In this assay, a wound or gap is created in a confluent monolayer of cells, and the closure of this gap is monitored over time. The rate of wound closure reflects the migratory ability of the cells. Similarly, this method has been employed in a previous study to evaluate the metastatic potential and migration of various cancer cell lines, including A549, HEPG2, and HCT116 [8]. The cytotoxic impact of DOX in this assay was particularly evident, as it caused cell dissociation, leading to an expansion of the wound area instead of closure, which suggests significant inhibition of cell migration. DOX effectively suppressed the ability of breast cancer stem cells to proliferate, migrate, and form colonies [23]. Conversely, ACT and PMS therapies resulted in a gradual reduction in migration over time. ACT exhibited a more substantial inhibitory effect on migration, while PMS showed a relatively lesser impact on the MCF-7 cancer cells. This suggests that ACT and PMS influence pathways critical to cell motility, thereby reducing the cells' ability to migrate and close the wound.

The study also focuses on the ability of these plant compounds to modulate apoptosis through the activation of Caspase 3/7. Apoptosis controls a number of cellular functions, including the removal of damaged or unnecessary cells from an organism, and it is usually regulated by Caspases, which are proteases that can cleave regulatory proteins involved in cell survival and maintenance [27]. For this reason, dysfunction of this cellular process, as well as the inactivation of these proteases, frequently leads to diseases such as cancer, which is an uncontrolled division and growth of cells. In the context of human breast adenocarcinoma, the MCF-7 cancer cell line has been found to be Caspase-3 deficient due to the partial deletion in the *CASP-3* gene [28], making it challenging to stimulate apoptosis naturally. Therefore, it is significant to consider how ACT and PMS may affect apoptosis in order to address the problem. The action of ACT on the breast cancer 4T1 cell line causes apoptosis by upregulating the expression of Caspase-3 [29], which has also been observed in the present study conducted as increase in the percentage of apoptotic cells were observed for both ACT and PMS. Furthermore, additional proteins involved in the apoptotic pathway were assessed and results have revealed an increase in the Bax/Bcl-2 ratio, suggesting an occurrence of apoptosis. Likewise,

for PMS, it has been found that it was able to enhance cleaved Caspase-3/9 activity and reduce the mitochondrial membrane potency in lung cancer cell line 95D, which is associated with apoptosis [30]. Moreover, it has also been observed that its interaction and inhibition with Akt and p38 MAPK phosphorylation could be considered responsible for its apoptotic regulation. In the present study conducted, findings suggest that upon treatment of the cancer cell with DOX, ACT, and PMS, an increase in the Caspase-3/7 activity was observed in the flow cytometric analysis, clearly showing the occurrence of apoptosis even in Caspase-3 deficient cells.

Network pharmacology is an emerging field that merges systems biology, pharmacology, and bioinformatics to elucidate on the complex relationships between disease-associated genes and drug targets, offering valuable insights for the development and discovery of novel therapeutic strategies [31]. This new approach shifts the focus of drug discovery from designing highly selective ligands for individual drug targets to identifying network-based targets, thereby enabling the development of multicomponent therapies [32]. Numerous studies have employed this *in silico* approach to uncover the therapeutic potential of new compounds, particularly in cancer research [33, 34]. Cancer progression is influenced not only by the cancer cells themselves but also by the surrounding tumor microenvironment. Network pharmacology, with its ability to map and analyze these complex interactions, offers a promising solution to this challenge, providing valuable insights into the multifaceted biological networks that drive tumor progression. In the present study, this approach facilitated the identification of key genes and proteins involved in various signaling pathways and mechanisms in breast cancer, with a particular focus on the targets of ACT and PMS. Results of the study have demonstrated that ACT and PMS target the protein coding genes - *PI3KR1, STAT1*, and *TLR* - all of which are associated with cancer. A member of the lipid kinases, PI3K plays a major role in multiple physiological functions such as glucose metabolism, cell growth, proliferation, and survival [35]. This interacts with and helps activate other proteins like Akt, which in turn opens up downstream signaling cascades that lead to the increase of transcription and translation, protein synthesis, and cell cycle progression. In most cancer cases, the PI3K/Akt/mTOR pathway is commonly dysregulated [36]. For breast cancer, it was found that PI3K overexpression promoted tumorigenesis and disease progression [37]. This is often attributed to mutations in the PI3K alpha catalytic subunit (PIK3CA), which is the primary cause of pathway hyperactivation [38]. These mutations are commonly amplified in various malignancies [39], resulting in heightened PI3K activity which enhances the aggressiveness of the disease [40]. This activity would then transform normal cells, granting them the potential to become malignant, thus contributing to tumor formation [41]. STAT1 is another protein target of the plant metabolites that facilitates the expression of genes necessary for maintaining cell viability in response to different stimuli. However, its specific role in cancer is still uncertain as this could act both as a tumor suppressor and as an oncoprotein. In fact, a study found that some cancer patients with elevated STAT1 levels had a positive prognosis [42]; however it was also observed that increased mRNA levels of STAT1 is associated with poor prognosis and tumor survival in breast cancer [43]. A particular study has identified that elevated protein expression of STAT1, along with high levels of CD74, characterized a subtype of triple-negative breast cancer (TNBC) associated with greater invasive and metastatic potential [44]. In addition to these proteins, another target of ACT and PMS is TLR, which is involved in the innate system of the body and allows the activation of transcription factors during the invasion of pathogens. In the case of cancer, these TLRs are seen to be overexpressed in cancer and its activation often results in aggressive progression of the disease [45]. Moreover, the interaction between TLR and other signaling pathways in cancer allows for the secretion of inflammatory cytokines and chemokines which promotes cell proliferation and apoptosis resistance [46]. In breast cancer, the TLR1-TLR10 receptors were found to be expressed in the MDA-MB-231 breast cancer cell line, with TLR4 being notably overexpressed. Silencing the *TLR4* gene resulted in decreased levels of IL-6 and IL-8, subsequently reducing the viability of the breast cancer cells [47].

The KEGG pathway analysis of the study has also supported these proteins' involvement in cancer as it has demonstrated that they affect the *PD-L1* expression and the PD-1 checkpoint pathway in cancer. In breast cancer, *PD-L1* expression is a key factor in immune evasion, with levels varying based on tumor stage and molecular subtype [48]. Given its role in cancer progression, *PD-L1* has become a critical target for immunotherapy. One key regulator of *PD-L1* expression is the PI3K pathway, which has been shown to promote *PD-L1* expression in various cancers. For instance, in a specific study on melanoma cells, treatment with a PI3K inhibitor led to a reduction in *PD-L1* expression [49], suggesting that PI3K signaling plays a role in modulating immune responses by influencing *PD-L1* levels. Similarly, STAT1 has also been linked to *PD-L1* expression, as demonstrated in ovarian cancer, where STAT1 knockdown resulted in decreased *PD-L1* expression [50]. Furthermore, TLR signaling is correlated with *PD-L1* overexpression, contributing to poor prognosis in peripheral T-cell lymphomas [51]. In summary, the *PD-L1* expression and PD-1 checkpoint pathway plays a critical role in both the survival and progression of cancer because the interaction between PD-1 and *PD-L1* results in T cell and interleukin malfunction, which often results in tumor progression [52]. Furthermore, *PD-L1* allows tumor cells to have a immunosuppressive microenvironment as well as resistance to cytotoxic T cell (CD8+) mediated cell killing [53]. As a result, several medications have been created to inhibit this pathway and for cancer immunotherapy, including pembrolizumab and anti-PD-1/*PD-L1* antibodies [54].

The *in vitro* analysis findings show that ACT and PMS have potential anti-cancer effects against MCF-7 breast cancer cells. These results call for investigation into the molecular pathways that underlie these plant metabolites'

actions. Heading into the area of *in silico* analysis, it was revealed that an intricate connection was observed between ACT, PMS, and key proteins involved in the development of cancer. First, this study shows that the main targets of ACT and PMS are PI3K, STAT1, and TLR which are involved in facilitating cancer cell invasion and metastasis; and are linked with the *PD-L1* expression and the PD-1 checkpoint pathway in cancer. The congruence of the *in vitro* observations with the *in silico* results on the inhibition of cell migration and invasion by ACT and PMS points to a plausible mechanism by which these metabolites prevent the progression of cancer.

Author Contribution Statement

KAC, LJC, DJS, KM, and MKD performs *in vitro* cell culture experiments and processing of data. KAC, LJC, DJS, JP performs in silico processing of the compounds. All authors (KAC, LJC, DJS, KM, JP, MKD, MD) are involved in the analysis of data, manuscript preparation, and its revision. JP, MKD, and MD contribute on the conceptualization of the study.

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Approval

This study is an approved student thesis by the Department of Biochemistry, Faculty of Pharmacy, University of Santo Tomas, Manila

Data Availability

Raw and supplementary data is available and can be requested to Kyle Andrei Cabatit at kyleac.cabatit@gmail.com.

Conflict of Interest

The authors declare no conflict of interest

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