ARTICLE

Anti-proliferative activity of leaf extracts from Antipolo (*Artocarpus blancoi* (Elmer) Merr.)

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ABSTRACT

The discovery and isolation of bioactive compounds from medicinal plants opened a new avenue in pharmacology, opening new avenues for cancer treatment. Most of these bioactive compounds are known to affect the hallmarks of cancer such as unrestrained proliferation. This study aims to assess the effect of crude leaf extracts (methanolic, aqueous, ethyl acetate and hexane extracts) from *Artocarpus blancoi*, locally known as "Antipolo," on cancer cell proliferation. The MCF-7 cell line was used to screen Antipolo's cytotoxic activity. However, although MCF-7 is widely used for breast cancer research, it has limitations because it lacks certain mutations found in more aggressive breast cancer subtypes. Crude leaf extracts of *A. blancoi* were tested for effects on MCF-7 cell viability using an MTT assay and Ki67 immunofluorescence. Phytochemical analysis identified alkaloids, anthraquinones, flavonoids, steroids, and tannins in the leaves of *A. blancoi*. The ethyl acetate extract was the most cytotoxic (IC50 = 47.2 µg/mL). Immunofluorescence staining for Ki67 showed that Ki67 expression decreased at lower concentrations (25 and 12.5 µg/mL) of the ethyl acetate extract. Altogether, these results suggest that the ethyl acetate extract from *A. blancoi* may possess potential as a therapeutic agent in breast cancer treatment.

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INTRODUCTION

Cancer is defined as a genomic disease at the cellular level (Pecorino 2012; Davidson 2016; Abotaleb et al. 2018). Without a definitive etiology, researchers listed factors contributing to cancer cell mutation and progression. These factors are characterized by specific traits known as hallmarks. These hallmarks are fundamental principles that outline the mechanisms by which a

KEYWORDS

MCF-7, cell proliferation, leaf crude extracts, MTT assay, breast cancer, medicinal plant

normal cell can transform into a malignant one (Baldassarre and Belletti 2016; Aftimos et al. 2019). In addition, these hallmarks provide insights into the complexities of cancer, enhancing our understanding of cancer biology and guiding the development of effective treatments. Recent molecular studies of cancer provide insights into disease progression (Baldassarre and Belletti 2016). Signaling pathways that regulate gene and protein expression are directly involved in proliferation, cell cycle, apoptosis, and angiogenesis, and these pathways are the focus of intensive study. These factors are often considered the key players in the process of carcinogenesis that shed light on how cancer develops (Omori et al. 2011; Pecorino 2012; Baldassarre and Belletti 2016; Aftimos et al. 2019). For instance, the proliferation marker Ki67, a non-histone protein found in the nucleus during the phases of the cell cycle (Villacorte et al. 2013; Mannell 2016b), is commonly used in determining the percentage of cancer cell proliferation (Sagar et al. 2006; Inwald et al. 2013).

Breast cancer is the most prevalent cancer in women and the second most common cancer globally (Nabet et al. 2017; International WCRF 2018). In the Asian region, 2015 statistics revealed that the Philippines had the highest number of new breast cancer cases and that it was the leading cause of death among Filipino women, with 20,267 new cases 7,384 documented deaths, accounting for 23% of all cancer-related fatalities (Laudico et al. 2015).

However, conventional cancer treatments often cause severe side effects (Pecorino 2012). This has prompted calls for alternative, less toxic therapies (Roy et al. 2017). This has led several researchers to shift focus to the potential use of natural products derived from medicinal plants which have long been employed as traditional remedies for various ailments (Greenwell and Rahman 2015). Medicinal plants contain bioactive compounds (e.g., flavonoids, alkaloids, phenolics) that can be isolated from various plant parts. These compounds can inhibit cancer cell proliferation, laying the foundation for other pharmacological studies (Solowey et al. 2014; Altemimi et al. 2017). One group of medicinal plants endemic to the Philippines is the genus *Artocarpus*, which is known to be rich in phenolic compounds, including flavonoids, stilbenoids, and others (Jagtap and Bapat 2010). Among the species in this genus, *Artocarpus blancoi*, which is endemic to the Philippines, has no previously reported data on its biological activity. Hence, this study investigated the effects of crude leaf extracts (methanolic, aqueous, ethyl acetate, and hexane) from *A. blancoi* (Antipolo)—an endemic Philippine plant—on cancer cell proliferation, a hallmark of cancer. These findings may provide insights into using Antipolo in cancer therapy and contribute to the knowledge of Philippine medicinal plants."

MATERIALS AND METHODS

Plant Collection

Artocarpus blancoi leaves (Supplemental Figure 1) were collected from Barangay Suarez (Supplemental Figure 2) in Iligan City, Lanao del Norte, Philippines. Specimens were morphologically identified and verified by a botanist from the Department of Biological Sciences, MSU-IIT, Iligan City. A voucher specimen (USTH 018-472) was deposited at the University of Santo Tomas Herbarium.

Methanolic crude extract preparation

The extraction and partitioning protocols were adapted from Altemimi et al. (2017) with modifications. The leaves were air-dried for two weeks. Then, the dried leaves were ground into a fine powder and placed in a jar. The powder (1 g) was soaked in 3 mL of absolute methanol and kept in the dark for three days. The methanol extract was filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator.

Hexane and ethyl acetate solvent partitioning

100 mL of the methanol extract was placed in a separatory funnel with an equal volume of ethyl acetate. When the two (2) solvents were partitioned, the methanolic layer partition at the bottom of the funnel was collected and mixed with 95% n-hexane until another partition was visible. The process was repeated three times, and all extracts of each solvent were combined. The combined extracts were concentrated under vacuum (35–40°C) and further dried at 35 °C.

Preliminary Screening of Phytochemical

The methanolic crude extract from the leaves of *A. blancoi w*as submitted to the Chemistry Department, College of Science and Mathematics, MSU-Iligan Institute of Technology, Iligan City, Philippines, for phytochemical analysis using standard methods with modifications (Subebe *et al.*, 2024).

In vitro cytotoxicity assay

a. Cell culture maintenance

Epithelial breast cancer cell line (MCF-7) was procured from the American Type Culture Collection, ATCC (Manassas VA, USA) and preserved with the cell culture collection in the Mammalian Cell Culture Laboratory, Institute of Biology, University of the Philippines-Diliman, Quezon City, Philippines. The MCF-7 cell line was cultured in T25 culture plates in Minimum Essential Medium (MEM) (GIBCO, California, USA) at 37oC under 5% CO_2 and 100% humidity with 10% Fetal Bovine Serum (GIBCO, California, USA), and 1% gentamicin antibiotics and Gibco 1% Insulin Transferrin Selenium (GIBCO, California, USA). Passaging occurred when the cells reached around 90% confluence in the culture plates. The cells were detached from the plates using 0.2% trypsin-EDTA (Invitrogen, California, USA) and transferred to new plates. Fresh medium was added to neutralize trypsin-EDTA and the cells were allowed to proliferate.

b. MTT assay

MCF-7 cells were seeded at a density of 4 x 10^4 cells/mL in sterile 96-well culture plates and incubated overnight at 37°C and 5% CO_2. Ten (10) mg samples of *A. blancoi* crude extract (methanol, ethyl acetate, hexane, and aqueous) were weighed using an analytical balance and placed in a 2 mL Eppendorf tube. The collected samples were dissolved using dimethyl sulfoxide (DMSO) with a stock concentration of 10,000 µg/mL. A master dilution plate (MDP) was prepared to dilute the stock concentration to the desired concentrations to be tested against MCF-7 breast cancer cells. Doxorubicin (DOXO), a chemotherapy drug from *Streptomyces peucetius var. caesius*, was used as a positive control (doses 0.078–10 µg/mL). Cells treated with the same volume of DMSO (no extract) were the vehicle control. After 72 h of incubation at 37°C and 5% CO_2, the media were then removed and replaced with 20 µL of 5 mg/mL MTT dye dissolved in phosphate-buffered saline (PBS) in each well. The cells were incubated at 37°C and 5% CO_2 for 4 hours. To dissolve the formazan crystals, 150 µL of DMSO was added to each well and then the absorbance value was read using an LEDETECT reader at 570 nm. Absorbances (optical density or OD) were read at 570 nm wavelength using a spectrophotometric microplate reader. The percent inhibition per concentration was calculated using the following formula (Cruz 2022):

$$\%Inhibition = \left[\frac{OD\ of\ vehicle\ control\ DMSO - OD\ of\ sample}{OD\ of\ vehicle\ control\ DMSO}\right] x\ 100$$

Equation 1. Computation for percent inhibition using MTT assay.
The Inhibition Concentration 50 (IC_{50}) was determined by calculating the inhibition values against sample and control concentrations using GraphPad Prism 7. Three trials, each with duplicate measurements, were performed for each assay.

Immunofluorescence of Ki67

Immunofluorescence (IHC) was performed based on previous studies (Villacorte et al. 2013; Omori et al. 2011) with modifications; experiments were done in duplicate for two trials. For immunofluorescence, 3000 MCF-7 cells/well in 96-well plates were seeded in duplicate. After 72 h, the cells were washed three times in phosphate-buffered saline (PBS) and fixed in freshly prepared 4% paraformaldehyde-PBS for 10 minutes at room temperature. Permeabilization was performed in PBS/Triton X-100 (0.2%) for 5 minutes at room temperature. Afterward, cells were washed three times in PBS, then exposed to the appropriate dilution, 1 µL:500 µL, of primary antibodies of Ki67 (rabbit polyclonal; PA5-19462, Invitrogen, California, USA) with 5% FBS in PBS overnight at 4ºC. Afterward, the cells were washed with 1x in PBS. Cells were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen A-11008) for 30 minutes at room temperature. Finally, the nuclei were stained with Hoechst 33342 (H3570, Invitrogen, California, USA), which was then diluted with a ratio of 1 mL:2000 mL for 5 minutes; then the cells were washed with 1x PBS. Samples were then examined with a ZEISS fluorescence microscope (Baden-Württemberg, Germany). Ki67 expressing cells were counted using the ImageJ application and % proliferation was calculated using the formula:

$$Ki67\%\ proliferation = \frac{Ave.positive\ antibody\ x\ average\ number\ of\ cells\ (treated)}{Ave.positive\ antibody\ x\ average\ number\ of\ cells\ (control)} x100$$

Equation 2. Computation for percent proliferation.

Statistical analyses

Using SPSS software, a student's *t* test was used to compare the means of two groups, followed by an *F*-test. Analysis of Variance (ANOVA) was used to compare the means of three or more groups, followed by Bonferroni's test to correct for multiple comparisons. $p<0.05$ was significant for MTT antiproliferative assay.

RESULTS AND DISCUSSION

Phytochemical Screening of the crude extract of *Artocarpus blancoi*

The phytochemical screening results revealed the presence of secondary metabolites, including alkaloids, anthraquinones, flavonoids, steroids and tannins. Detected bioactive compounds can be visualized by certain levels of abundance, with a "+" sign indicating the presence of the active compound. A single plus (+) suggests a low level of abundance, a double plus sign (++) indicates a moderate level and a triple plus sign (+++) signifies a high level. Table 1 enumerates the detection levels of these compounds. It shows that flavonoids, steroids, and tannins had the highest detection level, with a triple plus sign (+++) in the leaf of *A. blancoi*, thus, indicating its abundance. In contrast, alkaloids had a moderate detection level and anthraquinones had the lowest detection level.

Table 1: Phytochemical screening of the crude methanolic extract of *Artocarpus blancoi* indicating its phytochemical constituents with its level of detection, high detection (+++), moderate detection (++), low detection (+).

Sample	Alkaloids	Anthraquinones	Flavonoids	Steroids	Tannins
A. blancoi	(++)	(+)	(+++)	(+++)	(+++)
Remarks: (+++) – presence is rich or very rich. (++) – presence is in moderate amounts. (+) – presence is very poor					

The phytochemical assessment was performed to provide a baseline knowledge of the secondary metabolites in the leaves of *A. blancoi*, an endemic plant in the Philippines (The Plant List 2013). The researchers found that flavonoids, tannins and steroids are highly detected from the extract, followed by alkaloids with a moderate detection level and anthraquinones with the lowest detection level. Previous studies have shown that flavonoids, tannins and steroids have anti-proliferative and cytotoxic properties (Darvin et al. 2015; Abotaleb et al. 2018; Havsteen 2022). Moreover, these compounds interfere with multiple signaling pathways (*e.g.*, inducing G1/S arrest and mitochondrial apoptosis in gingival cancer cells) (Ravishankar et al. 2013; Darvin et al. 2015). Previous studies reported that alkaloids also play an important role as an anti-cancer agent by inducing apoptosis (Mohan et al. 2012; Kumar and Pandey 2013). At the same time, anthraquinones possess a broader spectrum of important biological activities such as antimicrobial, anti-inflammatory, anti-cancer and phytoestrogen activities (Chien et al. 2015; Malik and Muller 2016; Sharma et al. 2017). Based on these studies and the results from phytochemical screening, we investigated whether crude leaf extract from *A. blancoi* would show an anti-proliferative activity against breast cancer cell line MCF-7.

Cytotoxic activity of different extracts of *A. blancoi* against MCF-7

Effects on MCF-7 cells were assessed after 72 h exposure to *A. blancoi* leaf extracts at 6.25–100 µg/mL. The MCF-7 cell line, derived from human breast adenocarcinoma, is widely used in cytotoxicity and antiproliferative assays, including evaluating the effects of plant extracts. However, despite its popularity, this cancer cell line does not reflect the diversity of breast cancer subtypes (e.g. triple-negative or HER2+) which represents the luminal A-type breast cancer type (Neve et al. 2006; Holliday and Speirs 2011). Thus, these cancer cells are non-invasive and do not metastasize. Our findings revealed that the aqueous, methanolic, and hexane crude extracts showed no significant cytotoxic activity against the MCF-7 cancer cells, even at a concentration of 100 µg/mL (Figure 1). In contrast, the ethyl acetate extract caused the greatest decrease in viability: at 100 µg/mL, cell viability was only

18.18 ± 0.014% of control. The ethyl acetate extract was the most cytotoxic (IC50 = 47.2 µg/mL).

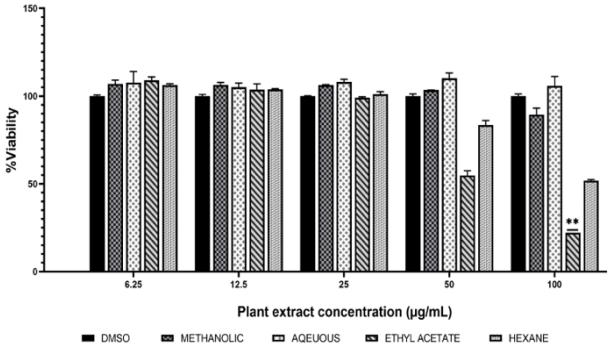

Figure 1: Percent viability of MCF7 cell line treated with DMSO, aqueous, methanolic, ethyl acetate, and hexane extracts. ** significant at p<0.05.

The cytotoxic effect of the different concentrations of ethyl acetate extracts against MCF-7 cells is also evident in the observed morphology of the MCF-7 cells in the vehicle control versus that of cells treated with positive control doxorubicin (DOXO) (Figure 2). In Figure 2A, control MCF-7 cells reached full confluence and maintained their typical epithelial morphology. After 72 hours, cells treated with DOXO became smaller and clumped (Figure 2B). Similar irregular, clumped morphology was observed in cells treated with 100 µg/mL ethyl acetate extract (Figure 2C).

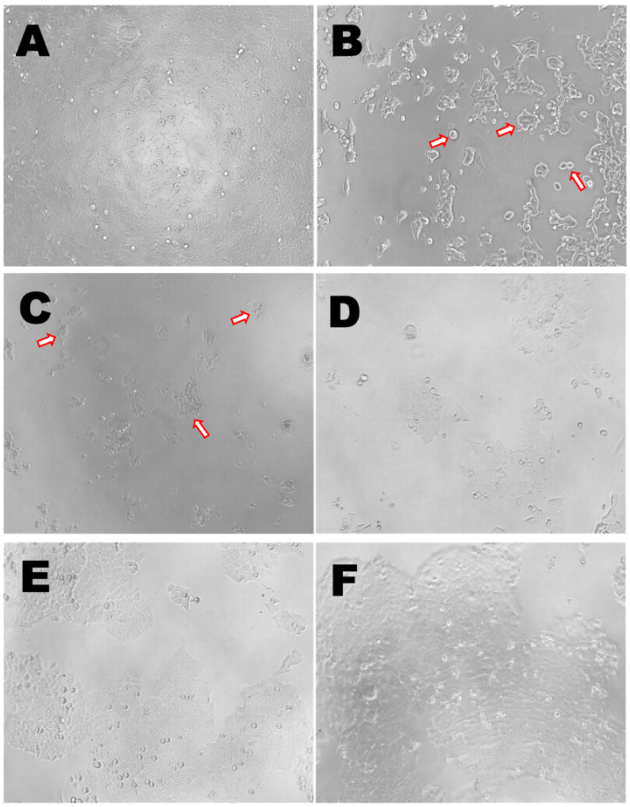

Figure 2: Microscopic images showing the cell population confluency and morphology of MCF-7 cell line. (**A**) Untreated cells exhibited epithelial morphology. (**B**) Doxorubicin (DOXO) shows clumped cells with irregular shape (arrows). Cells treated with ethyl acetate leaf extract of *A. blancoi* (**C**) 100 µg/mL show similar phenotype exhibited in DOXO (arrows); (**D**) 50 µg/mL (**E**) 25 µg/mL (**F**) 12.5 µg/mL concentrations displayed no changes in their morphology.

According to the US NCI classification for plant bioactive compound activity level, plant extracts with an IC50 value greater than 30 µg/mL but less than 100 µg/mL are considered moderately active (Canga et al. 2022). Based on our results, ethyl acetate extract from *A. blancoi* is moderately active.

To further elucidate the effect of *A. blancoi* leaf extracts in the proliferation of MCF-7 cells, we conducted immunofluorescence staining of the proliferation marker Ki67, utilizing the ethyl acetate extract as it exhibited the most cytotoxicity against MCF-7 cells. Furthermore, we specifically chose the 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL concentrations to determine the possible sublethal effect in the proliferation of MCF-7 cells.

Ki67 expression in MCF-7 treated with *A. blancoi* crude leaf extract

The proliferation marker, Ki67, is a nuclear antigen and forms an integral part of cell division in both standard and malignant tissues (Soliman et al. 2016; Miller et al. 2018). One of the hallmarks of cancer is its uncontrolled and relentless cell proliferation, which makes the Ki67 proliferation index commonly used to assess cancer (Abdel-Salam et al. 1984; Gerdes 1984). Figure 3 shows the expression of Ki67 in MCF-7 cells treated with ethyl acetate extract from *A. blancoi* and the vehicle control (DMSO). In this figure, Hoechst is colored blue, while Ki67 is colored green and these two colors are merged to identify the level and location of the expression of Ki67 in MCF-7 cells. Ki67 expression was observed in almost all MCF-7 cells of the DMSO control (Figure 3A). At 25 µg/mL of ethyl acetate extract, the percent proliferation of MCF-7 cells is 102.2%, suggesting that these cells are actively proliferating (Figure 4). At 12.5 and 6.25 µg/mL, Ki67-positive cells decreased to 76.8% and 71.2% of control, respectively, consistent with reduced proliferation (Figure 3B; Supplemental Fig. 3A-B).

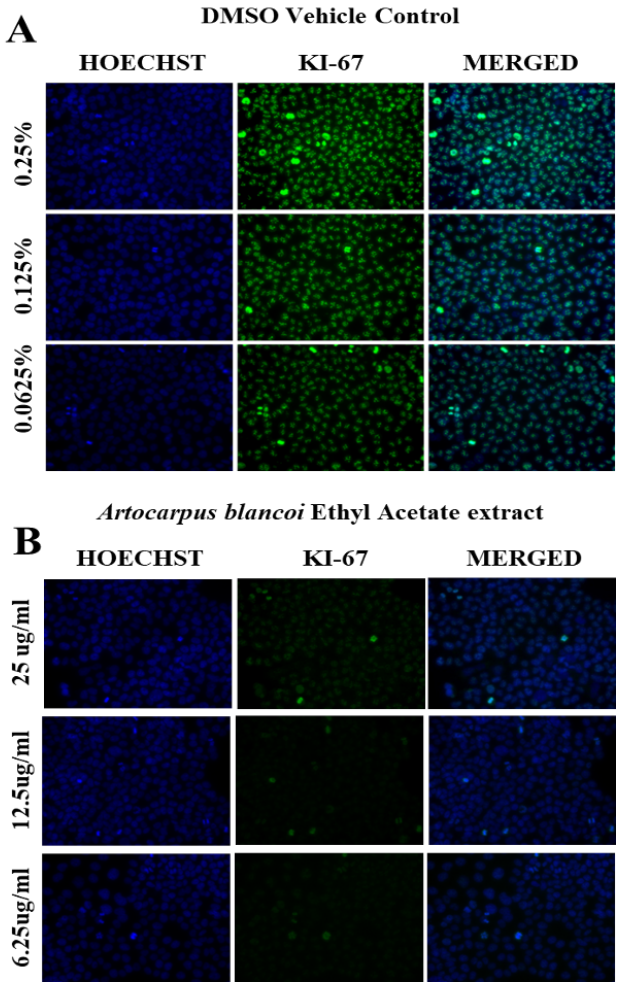

Figure 3: Immunofluorescence of Ki67 in MCF-7 cells treated with ethyl acetate *A. blancoi* leaf extract. (**A**) Ki67 are expressed in all cells treated with the vehicle control, DMSO. (**B**) No change of expression of

Ki67 at 25 µg/mL concentration of ethyl acetate. Ki67 expression is decreased at lower concentrations of ethyl acetate (12.5 µg/mL and 6.25 µg/mL).

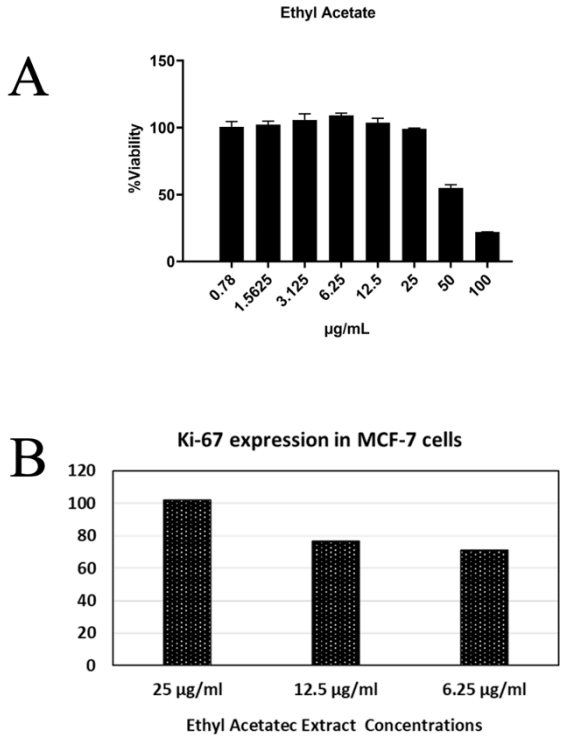

Figure 4: Graphs showing the: **A**. % viability of cells treated with ethyl acetate extracts. **B**. % proliferation of active Ki67 expression in MCF-7 cells treated with ethyl acetate extracts.

Ki67 immunostaining showed a dose-dependent decrease in proliferation: at 25 µg/mL, proliferation was 102.2% of control; at 12.5 µg/mL it was 76.7%; and at 6.25 µg/mL it was 71.3% (Figure 4). At 25 µg/ml, the percentage of proliferation remains at 102.2%. Interestingly, we observed a decreased expression of Ki67 as the concentration decreased, which at 12.5 mg/ml resulted in 76.7% proliferation and at 6.25 mg/ml, a decrease of 71.3% proliferation. The Ki67 protein is observed to be abundant and active during cell division (Manell 2016). It is considered one of the star players during the proliferation of cells, and thus, in cancer cells with uncontrolled proliferation, Ki67 activity is expected to be highly active (Gerdes 1984; Miller et al. 2018).

Previous studies similarly report that certain phytochemicals decrease Ki67 activity, correlating with reduced proliferation in MCF-7 cells (Yonehara et al. 2015; Yu et al. 2015; Kubatka et al. 2016; Kapinova et al. 2018). Moreover, one group found that plant extracts such as young barley and oregano showed similar results in which Ki67 expression decreases in rat tumor cells (Kubatka et al. 2016). These findings indicate that plant-derived bioactives can inhibit Ki67 accumulation, thereby blocking cell division. Other studies suggest that ethyl acetate plant extracts at sublethal doses can induce cancer cell dormancy (Rahman et al. 2017; Vafaee et al. 2019; Elmore et al. 2022). Our study therefore examined Ki67 to assess whether *A. blancoi* extracts might similarly induce dormancy in MCF-7 cells. Our results showed Ki67-positive cells at 76.7% and 71.3% of control for 12.5 and 6.25 µg/mL, respectively. These findings are consistent with reports that plant bioactives inhibit proliferation markers (like Ki67) at sublethal concentrations.

Overall, the ethyl acetate extract appears to induce cell death at higher concentrations (50–100 µg/mL) but inhibit division at lower concentrations (6.25–12.5 µg/mL). At the lower doses, cells remained metabolically active but did not proliferate, suggesting induction of dormancy. Cell dormancy can be classified as (1) senescence, or irreversible cell cycle arrest, and (2) quiescence, a reversible cell cycle arrest (van Velthoven and Rando 2019). Inducing dormancy is being explored as a way to prevent metastasis and inhibit cancer progression (Prasanna et al. 2021). This strategy could allow lower chemotherapy doses and facilitate surgical removal of dormant tumors. These findings indicate active compounds in the *A. blancoi* ethyl acetate extract. We recommend future work to purify these compounds, clarify their mechanisms of action, and include normal cell lines in bioactivity studies.

CONCLUSION

This study provides the baseline scientific evidence demonstrating the anti-proliferative potential of Artocarpus blancoi (Antipolo), an endemic Philippine medicinal plant, against breast cancer cells. Phytochemical screening revealed that the leaves are rich in flavanoids, tannins and steroids, compounds widely associated with anticancer activity. Among the tested plant crude extracts, the ethyl acetate extract exhibited the strongest cytotoxic effect against MCF-7 breast cancer cell line. Morphological changes and MTT assay results confirmed concentration-dependent cytotoxicity at higher doses, while immunofluorescence analysis demonstrated a reduction in Ki67 expression at sublethal concentrations, indicating suppression of cell proliferation rather than immediate cell death. In addition, the observed decrease in Ki67 expression at lower cocnentrations suggests that the ethyl acetate extract may induce proliferative arrest or dormancy-like state in MCF-7 cells, a mechanism recognized as a promising strategy to limit tumor progression and metastasis. Thus, these findings highlight A. blancoi as a promising source of bioactive compounds with potential therapeutic relevance in breast cancer treatment. Further studies are needed to isolate and characterize the active components, elucidate their molecular mechanism of action, and evaluate their selectivity and safety using normal cell lines and additional breast cancer subtypes.

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CONFLICT OF INTEREST

The author declares that there are no conflicts of interests.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

RVB, RJA, MJBS, MMEM, SDJ, AO, LA, CCS, MMU, ARM, MVT helped with the conception, design of the study, the acquisition of data, and analysis and the interpretation of data. RVB and MVT were responsible for the writing of the original and revision of the manuscript.

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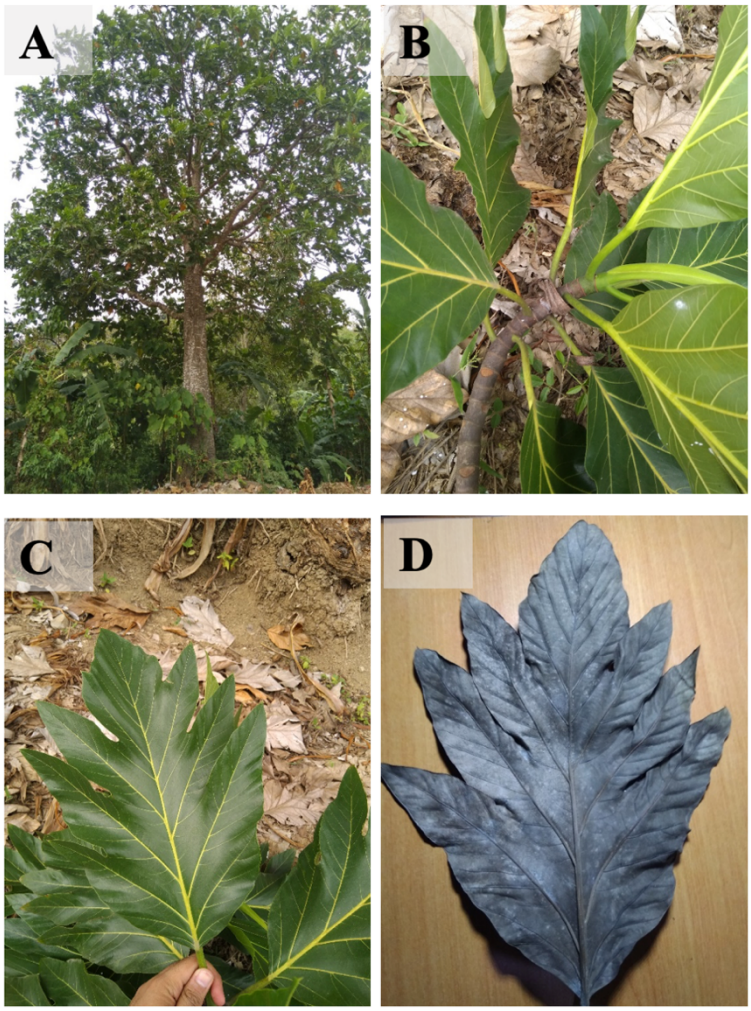
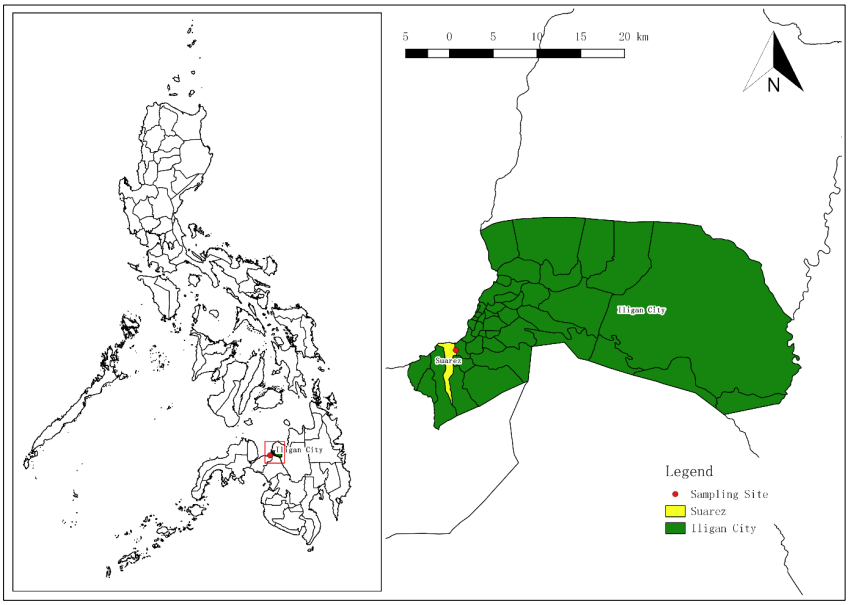
SUPPLEMENTARY FIGURES



Supplemental Figure 1: Photographs of *Artocarpus blancoi* (Elmer) Merr. at Barangay Suarez, Iligan City, Lanao del Norte, Philippines. (**A**) whole tree image of *A. blancoi*; (**B**, **C**, **D**) Leaf arrangement which is observed to be simple and spirally arranged.



Supplemental Figure 2: Shows the map of the collection site of the leaves of *Artocarpus blancoi* (Elmer) Merr. (**A**) shows Iligan City, Philippines; (**B**) shows the exact location of the sampling site in Suarez, Iligan City, depicted by red pointer.