Cytotoxic and Apoptotic Properties of the Flavonoid-rich Ethyl Acetate Fraction of the Crude Methanol Leaf Extract of *Syzygium samarangense* (Blume) Merr. (Myrtaceae) against MCF-7 Breast Cancer Cells

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ABSTRACT

Introduction. Breast cancer is the most common cancer among women in the Philippines and about 3 in every 100 Filipina will be diagnosed with breast cancer in their lifetime. There is a need to discover safe, yet inexpensive herbal extracts with potential cytotoxic properties as potential treatment modalities to treat breast cancer.

Objectives. This study seeks to explore the cytotoxic and apoptotic properties of the ethyl acetate fraction of the defatted crude methanol leaf extract of *Syzygium samarangense* in MCF-7 breast cancer cell lines.

Methods. Screening for flavonoids of the extracts was performed using TLC, total flavonoids, total phenols, FTIR and LC-MS spectroscopy. The hydrogen peroxide and ferric reducing anti-oxidant power were used as substrates to assess *in vitro* anti-oxidative properties of the extracts. The MTT dye viability assay was used to assess the cytotoxic properties of the extracts against MCF-7 cells. Apoptotic properties of the extracts in MCF-7 cells were determined by caspase-3 activation assay, DNA fragmentation patterns and fluorescence microscopy after annexin-V and propidium iodide staining.

Results. The abundance of flavonoids in the ethyl acetate fraction of the crude methanol leaf extract was established by TLC, FTIR, LC-MS/MS, total flavonoid and total phenol analyses. The *in vitro* anti-oxidative properties of this extract was comparable to ascorbic acid. The median inhibitory concentration (IC_{50}) of this extract in MCF-7 breast cancer cell lines was 7.2 mcg/mL while doxorubicin registered an IC_{50} of 1.2 mcg/mL. At this concentration, the extract was not cytotoxic to normally-dividing breast epithelial cells. Cytotoxicity of the extract was mediated via apoptosis as demonstrated by DNA fragmentation, caspase-3 activation and fluorescence microscopic analyses.

Conclusion. The study shows that the flavonoid-rich ethyl acetate fraction of the crude methanol leaf extract of *S. samarangense* possesses potent apoptotic and cytotoxic properties against MCF-7 breast cancer cell lines at low concentrations.

Keywords: Syzygium samarangensi, makopa, MCF-7 breast cancer cell lines, cytotoxic, apoptotic

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INTRODUCTION

Globally, for several decades, women have been facing health challenges pertaining to breast cancer. In fact, it is considered next to skin cancer in terms of the most prevalent cancers and affects women aged 50 and above. The statistics in 2021 validates this as breast cancer accounts for 12% of all new cases of cancer worldwide on an annual basis as recorded by WHO. Currently, about 287,850 new cases have been reported in women in the U.S. alone. At the end of 2022, about 43,250 women in the U.S. are expected to die from breast cancer.[1] In 2020, breast and cervical cancers are the top 2 leading malignancies affecting Filipino women, most of whom have been diagnosed at advanced stages.[2] A meta-analysis reports that 26 out of 100 females may be diagnosed with breast cancer in the Philippines. The DOH website cites the 2020 data from the Global Cancer Observatory, showing breast cancer as the leading cause of cancers and the third leading cause of cancer deaths among Filipinos.

In breast cancer chemotherapy, killing tumor cells is difficult as healthily dividing cells are affected due to non-selective drug targeting. This damage to normal and healthy cells are responsible for adverse effects associated with anticancer drugs. Several plant extracts and their secondary metabolites have been studied to exhibit cytotoxicity against breast cancer cells and, thus, offering complementary and alternative medicine since the cost of standard chemotherapy is prohibitive. Herbal medicines have taken a niche in the prevention and treatment of breast cancer due to their efficacy, relative safety, and cost-effectiveness.[3]

Among the myriad of plants that exhibit anticancer activities, an indigenous plant known in the Philippines as "makopa" or *Syzygium samarangense* is known to exhibit anticancer properties. The ethanol seed and fruit pulp extracts, which are rich in chalcone and quercetin glycosides, are reported to be cytotoxic against SW-480 human colon cancer cell lines.[4] The methanol fruit extract possesses antiproliferative and apoptotic activities against A549 lung cancer cell lines.[5] Herbal medicines have the potential to augment the current treatment of breast cancer but the latest cancer guidelines in the European and American societies (i.e., ESMO and ASCO) have no statements yet regarding their use for the prevention or treatment of breast cancer.

To date, the anticancer properties of *S. samarangense* leaf extracts remain to be discovered. The dichloromethane leaf extract contains several chalcone derivatives as well as the triterpenes betulin, lupeol and sitosterol.[6] Chalcones and triterpenes have been found to demonstrate cytotoxicity against MCF-7 breast cancer cells.[7,8] Also, the ethyl acetate fractions of the methanol leaf extract exhibited *in vitro* antioxidative effects using DPPH as a substrate.[9] It was for these reasons that this extract was investigated in this study for its cytotoxic and apoptotic effects in MCF-7 breast cancer cell lines.

METHODS

Plant Collection and Extraction

About 1 kilogram of leaves of *S. samarangense* was collected from a vacant farm lot in Barangay Cobol, San Carlos City, Pangasinan, and washed with water to remove adhering dirt. Leaves were air-dried for 2 weeks and then pulverized using a Wiley mill. A voucher specimen of the plant was submitted to the Philippine Herbarium for verification and was given a repository number of 18-2335-1. Ground dried leaves were macerated exhaustively by cold percolation with methanol. Combined methanol extracts were defatted with exhaustion by partitioning (1:1 v/v) with n-hexane. The defatted methanol extract was concentrated using a rotary evaporator (Buchi R-220) at 45 ± 5°C to obtain a bluish-green syrupy mass. This crude extract was suspended in distilled water in a separatory funnel and partitioned with ethyl acetate (1:1 v/v) with exhaustion. Combined ethyl acetate fractions were dried by rotary evaporation at 45 ± 5°C followed by lyophilization (FSF 18-N) to obtain coarse green granules.

Phytochemical Screening

A 1 mg/mL solution of the lyophilized ethyl acetate fractions in methanol was subjected to TLC analysis by applying as bands in 1 x 6 cm^2 plates (Merck, silica 60 F_{254} plate), developed using ethyl acetate-toluene-formic acid (4:4:1), and visualized under UV light at 254 nm. Plates were then sprayed with $K_2Fe(CN)_6$ - $FeCl_3$ TS. Separation was monitored using quercetin in methanol (1 mg/mL) as a standard.[10]

The lyophilized sample in KBr pellets was subjected to FTIR spectral analysis (Perkin Elmer Spectrum 2 FTIR spectrometer 2012) which generated infrared spectrum from 550 to 4000 cm^{-1} for 20 scans and 4 resolutions. Similar sample was analyzed by LC-ESI-MS/MS spectral analysis (Waters Xevo G2-XS QT, MSE mode) where substances were separated with a 1.8µm, 2.1 x 100 mm ACQUITY HSS-T3 C_{18} column using a mobile phase consisting of a 1:1 v/v mixture of acetonitrile: 0.1% formic acid in hexane at 120°C at a flow rate of 0.5 mL/minute. The software used for data acquisition and data analysis was Analyst 1.5.2 (AB Sciex, Framingham, MA, USA). Data were processed using a Waters UNIFI Scientific Information System v1.8.1.073 Library which allows putative identification of separated molecules based on retention times, molecular weights, and fragmentation patterns.

Total Flavonoids and Total Phenols

A stock solution of quercetin in methanol (1 mg/mL) was serially diluted with methanol to arrive at standard solutions having concentrations of up to 128 mcg/mL. Exactly 1 mL each of stock solution of the crude methanol leaf extract of *S. samarangense* (CMLE-SS) and the lyophilized ethyl acetate fraction (LEAF-SS) in 2% aqueous DMSO and each

of the standard quercetin solutions, was diluted with 4 mL of distilled water in 10 mL volumetric flasks, followed by the addition of 0.3 mL of 5% $NaNO_2$. After 5 minutes, 0.3 mL of 10% $AlCl_3$ was added to each flask. After 6 minutes, 2 mL of 1M NaOH was added to each flask, with distilled water being used to dilute the volumes to 10 mL, with thorough mixing. Absorbance was read at 510 nm (Perkin Elmer Lambda 365) and the total flavonoid content was measured as quercetin equivalent (mg/g) using the formula:

$$\text{Total Flavonoid} = \frac{\text{C x 10 mL x 1 mg}}{\text{1000 mcg x 0.001 g}}$$

where C was the concentration of quercetin obtained by linear regression analysis of the calibration curve that plots quercetin concentrations and absorbance values.[11]

For total phenol quantification, a calibration curve was obtained by using aqueous tannic acid standard of up to 100 mcg/mL. Ten milligram each of CMLE-SS and LEAF-SS was diluted to 10 mL with methanol. Both 0.5 mL of standard solutions and samples were taken and mixed with 2.5 mL of 50% Folin–Ciocalteu TS and 2.5 mL of distilled water and allowed to stand for 5 minutes. Two mL aqueous sodium carbonate (7.5%, w/v) was added to each and the final mixture was shaken and then incubated for 15 minutes. The absorbance of all standards and samples were measured at 765 nm and the results expressed as milligrams of tannic acid equivalents per 100 mg of sample.[11]

In vitro Screening for Anti-oxidative Properties

The hydrogen peroxide scavenging and ferric reducing anti-oxidant power (FRAP) assays were used to assess anti-oxidative properties. One mL of each of CMLE-SS, LEAF-FS, and ascorbic acid in 2% aqueous DMSO (up to 128 mcg/mL concentrations) were mixed with 0.6 mL of H_2O_2 solution (40 mM in phosphate buffer pH 7.4). Absorbance was read at 230 nm after 10 minutes against a blank solution containing phosphate buffer without H_2O_2. Percentage inhibition of H_2O_2 scavenging was calculated using the following equation:

$$\text{\% Inhibition} = \frac{\text{Ao - Ac}}{\text{Ao}} \text{ x 100}$$

where Ac is the absorbance due to sample solutions while Ao is absorbance due to the control blank. The median inhibitory concentration (IC_{50}) was calculated by regressing % inhibition against sample concentration.[12]

The solutions used in the FRAP assay were: 300 mM acetate buffer pH 3.6, 20 mM $FeCl_3 6H_20$ and 10 mM TPTZ (2,4, 6-tripydridyl-s-triazine) in 40 mM HCl. Exactly 25 mL of acetate buffer, 2.5 mL of $FeCl_3$, and 2.5 mL of TPTZ were mixed to prepare the working solution which was heated to 37˚C prior to the experiment. Exactly 10 µL each of CMLE-SS, LEAF-FS, and ascorbic acid (1 mg/mL in 2% aqueous DMSO) were mixed with 300 µL of the working solution for 30 minutes in a 96-well plate and the absorbance were read for each well at 593 nm. For construction of the calibration curve, 5 concentrations of $FeSO_4.7H_2O$ (0.1, 0.4, 0.8, 1, 1.12 and 1.5 mM) were used and the absorbance was measured at 593 nm. FRAP values are indicated as weight (in gram) of $FeSO_4$ in 100 grams of extract.[12]

Cytotoxicity Screening

The conduct of this experiment, and the succeeding screenings for apoptosis, was approved by the Biosafety Committee of the College of Pharmacy, Virgen Milagrosa University Foundation (San Carlos City, Pangasinan) and was given a protocol number PH-2018-11. MCF-7 breast adenoma (ATCC # HTB-22) and normal human epithelial breast myoblast cell lines (ATCC # 600-010) were obtained from the Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City, cultured in Dulbecco's modified eagle medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 U/mL). Cell lines were seeded separately in 96-well plates at 2 x 10^3 cells/well and exposed to CMLE-SS, LEAF-FS and doxorubicin in 2% aqueous DMSO (i.e., up to 128 mcg/mL concentrations). Aqueous 2% DMSO was used as blank control. Plates were incubated for 72 hours at 37˚C with 5% CO_2, after which 20 µL of MTT solution in 2% aqueous DMSO (5 mg/mL) was transferred into each well. The plates were further incubated for another 4 hours after which the contents of each well were discarded. The formazan residues in each well were dissolved in 100 µL of 2% aqueous DMSO. The absorbance of the plates was read at an emission wavelength of 570 nm and an excitation wavelength of 630 nm (LogPhase 600). Analyses were done in 5 replicates per sample. The % inhibition was calculated, thus:

$$\text{\% Inhibition} = \frac{\text{Ao - As}}{\text{Ao}} \text{ x 100}$$

where Ao is the average absorbance readings obtained with the blank control while As_o is the average absorbance readings for the samples or doxorubicin. The IC_{50} was determined by regressing % inhibition against concentrations.[13]

Apoptosis Screening

MCF-7 cells were treated with the CMLE-SS, LEAF-FS, and doxorubicin at their previously obtained IC_{50} values, using aqueous 2% DMSO as a negative control. After 48 hours, both adherent and floating cells were harvested after being trypsinized. Cells were washed with PBS pH 7.4 followed by centrifugation at 12,000 rpm. The pellets were suspended in NP-40 lysis buffer (BioSource, containing 1% NP-40 in 20 mM EDTA and 50 mM Tris-HCl pH 7.5). After 15 minutes, cells were treated with sodium dodecyl sulfate (1%) and RNase (5 mg/mL) at 37˚C followed by digestion with proteinase K (1 mg/mL) for 5 hours at 56˚C. After addition of ¼ volume with 10 M ammonium acetate, DNA pellets were precipitated with 1 volume of ice-cold isopropanol, mixed thoroughly, and kept for 1 hour at -20˚C

after which the mixture was centrifuged for 20 minutes at 12,000 rpm. DNA pellets were washed with 200 µL of ice-cold 75% ethanol and air-dried for 10 minutes. Pellets were dissolved in loading buffer for separation by electrophoresis (SYBR Safe) in 1.5% agarose gels at 60 volts for 90 minutes, after which gels were observed under UV.[14]

Caspase-3 activities were checked using double antibody sandwiched ELISA (Thermo Fisher - Invitrogen). MCF-7 cells, prepared similarly as the cytotoxicity assay, were exposed to CMLE-SS, LEAF-FS, and doxorubicin at their previously obtained IC_{50} values in 2% aqueous DMSO, with the latter solvent considered as a negative control. Caspase-3 solution, prepared according to the instructional manuals provided, were exposed to the extracts or doxorubicin in 96-well plates. Each well was precoated with a biotin-conjugated antibody specific to the enzyme. Thereafter, avidin conjugated to horseradish peroxidase was added to each well and incubated for 24 hours at 37˚C with 5% CO_2. The enzyme-substrate reaction was terminated by the addition of H_2SO_4 TS and the color change was measured at 450 nm (LogPhase 600). Caspase-3 activity was calculated as folds over control by calculating the ratio of the absorbance values obtained with the extracts or doxorubicin to that of 2% aqueous DMSO.[14]

MCF-7 cells were seeded for 24 hours at 4 x 10^5 cells per well in a 96-well plate as confirmed by an automated cell counter (Countess 2). Each well was treated with CMLE-SS, LEAF-FS, and doxorubicin at their previously obtained IC_{50} values in 2% aqueous DMSO, with the latter solvent considered as a negative control. The plate was incubated for 48 hours at 37˚C with 5% CO_2. Cells were centrifuged at 2000 rpm for 5 minutes and then washed with phosphate buffer pH 7.4 twice. Cells were stained with annexin V and propidium iodide for 5 minutes after which, they were washed with ice-cold Hank's balanced salt solution (HBSS) twice. The pellets were resuspended in 100 µL HBSS and mounted on glass slides with cover slips and viewed under a fluorescent microscope. Stained green nuclei appeared as apoptotic cells while stained red nuclei appeared as necrotic cells. Apoptotic index was calculated as the % of apoptotic cells in the total number of tumor cells counted.[14]

Statistical Analysis

To calculate IC_{50} values, linear regression analyses were done by plotting using Graphpad Prism. Means were compared by 2-tailed t-test and analysis of variance. Analysis was done in 5 replicates, unless otherwise specified.

RESULTS

Phytochemical Screening

Figure 1 shows the presence of quercetin corresponding to the singular bands generated by the quercetin standard when the plates are viewed under UV 254 and when sprayed with $K_2Fe(CN)_6$ - $FeCl_3$ TS. The rest of the bands are flavonoid aglycones or their glycosides and other poly-

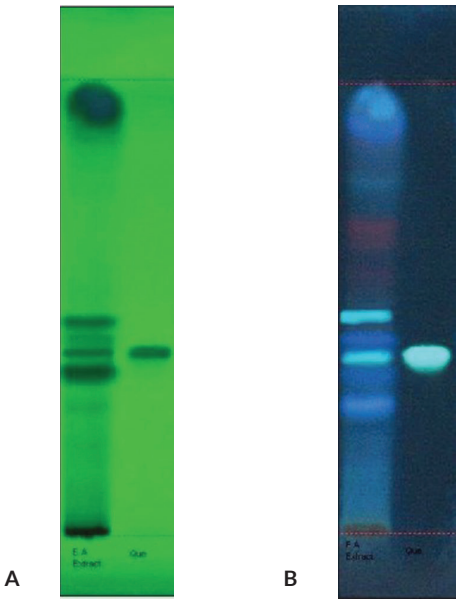


Figure 1. Thin-layer chromatograms of the Ethyl Acetate Fraction of the Crude Methanol Leaf Extract of *S. samarangense* at **(A)** UV 254 nm and with **(B)** $K_2Fe(CN)_6$ - $FeCl_3$ TS with Quercetin as Marker Substance on Right Lanes.

phenolic substances which fluoresce under UV light or appear as violet bands after derivatization with $FeCl_3$.

Figure 2 shows the FTIR spectra of the LEAF-SS extract and marker flavonoid quercetin. The absorption band at 1,600 - 1800 cm^{-1} is due to the ketone moiety of the benzopyrone ring. Aromatic hydroxyl groups are represented by 2 absorption peaks at 2,700 - 3,000 cm^{-1}. Intramolecular ether linkage in the flavone nucleus corresponds to small fingerprint absorption bands at 1,000 - 1,300 cm^{-1}. A broad absorption at about 1,700 cm-1 represents the aromatic rings.

Figure 3 shows 8 putative flavonoid substances separated from LEAF-SS using LC-ESI-MS/MS spectral analysis. These substances exist as aglycones or their corresponding glycosides and, thus, confirming the reported abundance of flavonoids in Figures 1 and 2. Previous literature reports on the abundance of other classes of flavonoids in *S. samarangense* leaves.[15]

Table 1 compares the total flavonoid and total phenolic contents of the crude methanol leaf extract of *S. samarangense* (CMLE-SS) and its lyophilized ethyl-acetate fraction (LEAF-SS) in addition to FRAP anti-oxidative properties. The greater number of phenolic substances quantified in LEAF-SS was due to semi-purification processes after the crude methanol extract was defatted with n-hexane.

Figure 4 compares the anti-oxidative effects of CMLE-SS and LEAF-SS with ascorbic acid in terms of H_2O_2 scavenging abilities.

The higher anti-oxidative potential observed with LEAF-SS is concurrent to its higher contents of phenolic

substances as observed in Figure 3 and Table 1. Anti-oxidative properties of flavonoids and related phenolic secondary metabolites are attributed to oxidative coupling of aromatic hydroxyl groups which, in this case, acts to reduce trivalent ferric cations to the divalent ferrous cation and prevent subsequent oxidation of H_2O_2 to O_2. Higher number of hydroxyl groups commensurate to greater free radical scavenging activities, a mechanism that correlates highly with possible antiproliferative properties in cancer cells.[16]

Figure 5 compares the cytotoxic effects of *S. samarangense* leaf extracts in MCF-7 breast cancer cells via antiproliferative mechanisms with CMLE-SS, LEAF-SS, and doxorubicin registering mean IC_{50} values of 87.3, 7.2 and 1.2 mcg/mL, respectively. Conversely, in Figure 6, the samples did not exhibit significant cytotoxicities in normally-dividing non-metastatic epithelial breast cells, where IC_{50} values were estimated at more than 1 mg/mL for both extracts, unlike doxorubicin which persisted to be cytotoxic in these cells.

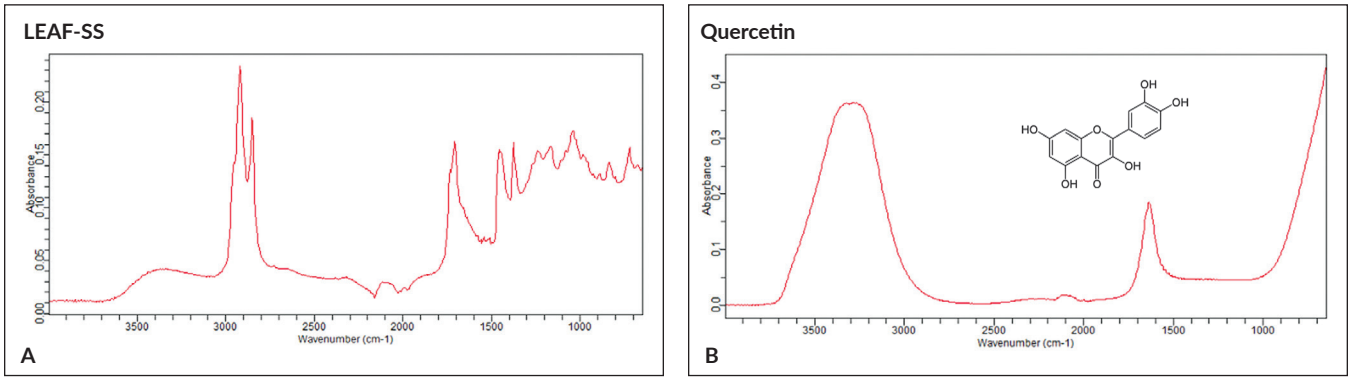
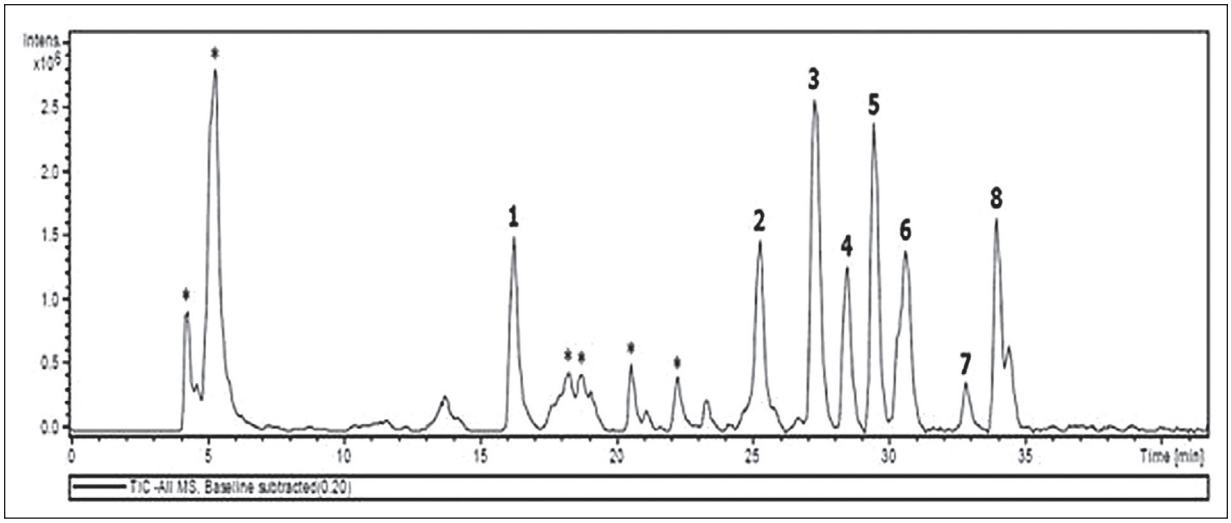


Figure 2. FTIR Spectrum of the Ethyl Acetate Fraction of the **(A)** Crude Methanol Leaf Extract of *S. samarangense* and the **(B)** Marker Substance Quercetin.



Legend: 1 – rutin, 2 – apigenin-O-rutinoside, 3 – quercetin, 4 – quercetin-3-O-glucoside, 5 – apigenin, 6 – luteolin, 7 – quercetin dimethyl ether 3-O-rurinoside, 8 – hesperidin

Figure 3. LC-EI-MS/MS Spectrum of the Ethyl Acetate Fraction of the Crude Methanol Leaf Extract of *S. samarangense*.

Table 1. Total Flavonoids and Total Phenol Contents of *S. samarangense* extracts

Name of Extract	Total Flavonoids (mg Quercetin / gram sample)	Total Phenol (mg tannic acid / gram sample)	FRAP Equivalent in gram of $FeSO_4$ per 100 grams of extract
CMLE - SS	46.3 ± 4.5 mg/g*	66.5 ± 6.4*	27.8 ± 2.9*
LEAF - SS	59.3 ± 3.1 mg/g	48.9 ± 4.7	45.7 ± 5.6
Ascorbic Acid	N/A	N/A	56.7 ± 3.9**

*n = 5; * p < 0.01 compared to LEAF-SS; **p < 0.001 versus both extracts*

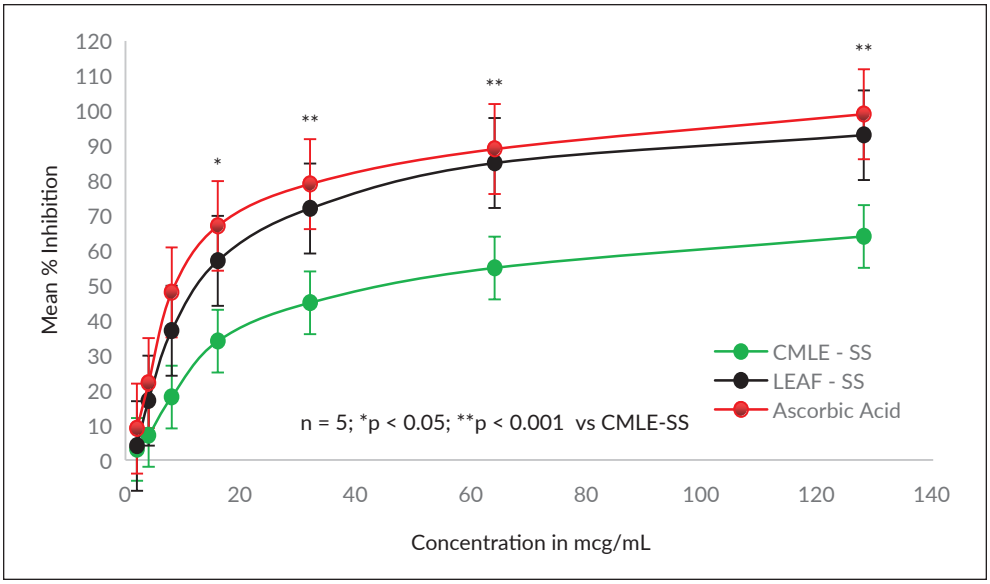


Figure 4. Comparative Concentration - H_2O_2 Scavenging Inhibition Curves Between the Crude Methanol Leaf Extract of *S. samarangense* and its Ethyl Acetate Fraction.

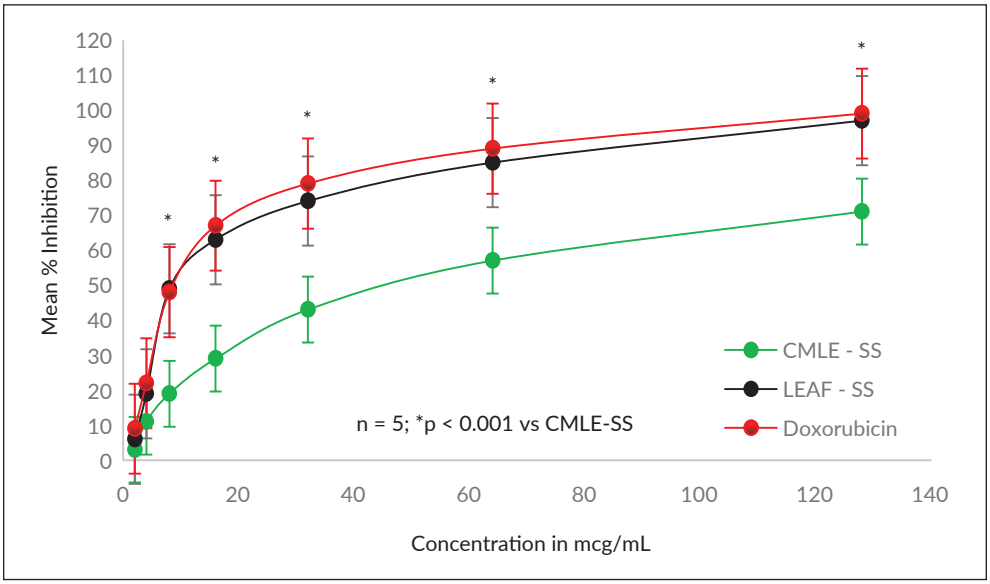


Figure 5. Comparative Inhibition - Concentration Curves of MCF-7 Breast Cancer Cell Lines Exposed to the Crude Methanol Leaf Extract of *S. samarengense* and its Ethyl Acetate Fractions.

According to the US National Cancer Institute, a plant extract exhibits significant *in vitro* cytotoxic activity against cancer cell lines after incubation for 72 hours if IC_{50} values are less than 20 mcg/mL.[17]

As evidence of apoptosis in MCF-7 breast cancer cells, Figure 7 compares the DNA fragmentation patterns between the 2 extracts of *S. samarangense*, in comparison with doxorubicin. Based on the reference molecular weight ladder in the first column, more DNA fragmentation occurred if cells were exposed to LEAF-SS (ladder 4 and 5) than when cells were exposed to CMLE-SS.

The effects of the 2 leaf extracts of *S. samarangense* on caspase-3 activation in MCF-7 breast cancer cells are tabulated and illustrated in Figure 8.

Activation of caspase-3 plays a central role in the execution phase of apoptosis for both intrinsic and extrinsic pathways, leading ultimately to chromatin condensation and DNA fragmentation. In this study, caspase-3 activation was

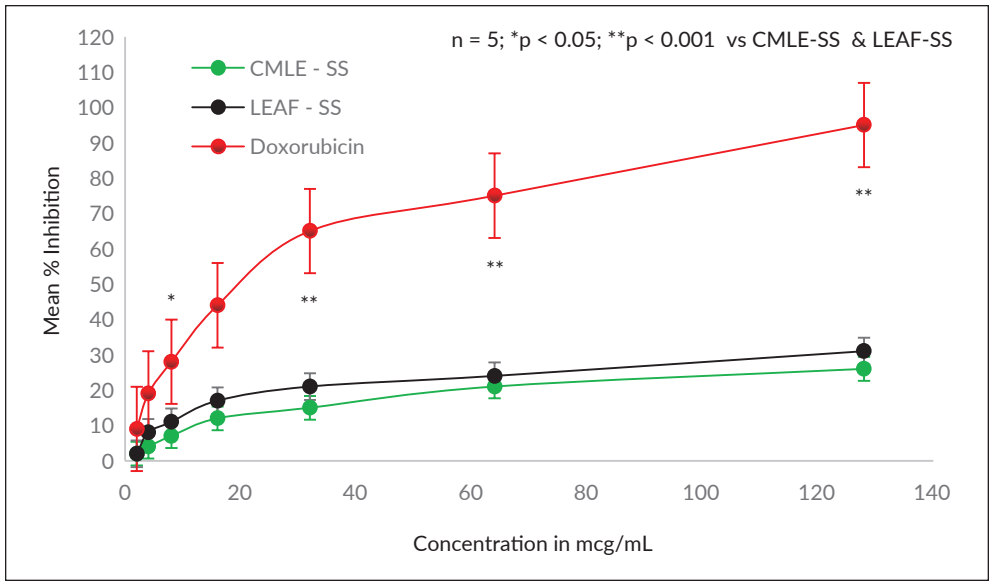


Figure 6. Comparative Inhibition - Concentration Curves of Normal Human Epithelial Breast Myoblast Cell Lines Exposed to the Crude Methanol Leaf Extract of *S. samarengense* and its Ethyl Acetate Fractions.

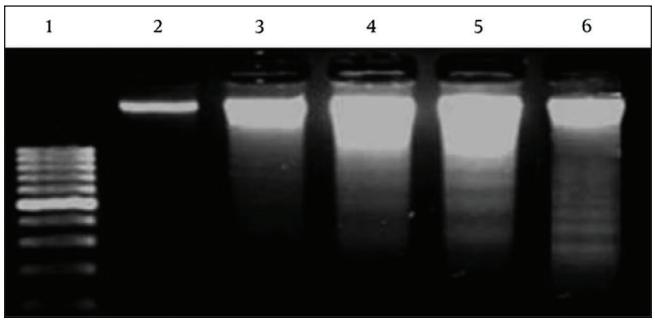
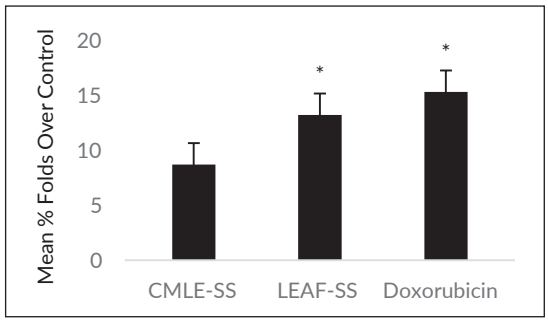


Figure 7. Electrophoresis Plate Showing DNA Fragmentation in MCF-7 Breast Cancer Cells Exposed to **(2)** 2% DMSO; **(3)** Crude Methanol Leaf Extract of *S. samarengense* and its **(4-5)** Ethyl Acetate Fraction and **(6)** Doxorubicin at IC_{50} Concentrations.

more significant when MCF-7 cells were exposed to LEAF-SS than when cells were exposed to CMLE-SS ($p < 0.001$). Figure 9 shows the activation cascades of caspase enzymes in both extrinsic and intrinsic apoptotic pathways of apoptosis, with capase-3 executing the signal for DNA fragmentation.[18]

Figure 10 compares the fluorescence micrograph of MCF-7 breast cancer cells that underwent apoptosis upon staining with propidium iodide and annexin V. In this case, higher apoptotic bodies were formed when cells were exposed to LEAF-SS at IC_{50}. The corresponding apoptotic indices for both extracts are also tabulated. The greater apoptotic indices obtained in MCF-7 cells obtained with LEAF-SS exposure ($p < 0.001$) over CMLE-SS exposure is congruent to the previous findings for DNA fragmentation and caspase-3 activation.



Sample at IC_{50}	Mean Absorbance	Mean % Folds Over Control
2% DMSO	0.012	N/A
CMLE - SS[a]	0.224	8.7 ± 1.9
LEAF - SS[b]	0.331	13.2 ± 2.3*
Doxorubicin[c]	0.371	15.3 ± 2.9*

*n = 5; *p < 0.001 vs CMLE-SS; [a] 87.3, [b] 7.2 and [c] 1.2 mcg/mL*

Figure 8. Caspase-3 Activation in MCF-7 Breast Cancer Cell Lines Exposed to the Crude Methanol Leaf Extract of *S. samarengense* at IC_{50} Concentrations.

DISCUSSION

MCF-7 is a highly metastatic and tumorigenic human breast adenoma cancer cell line with receptors for estrogen and progesterone hormones, making these cells retain several ideal characteristics particular to mammary epithelium such as cytoplasmic estrogen processing.[19] Quercetin, a flavonoid aglycone, was found to bind to and induce the expression of type-2 estrogen-binding sites in MCF-7 cells.[20] The

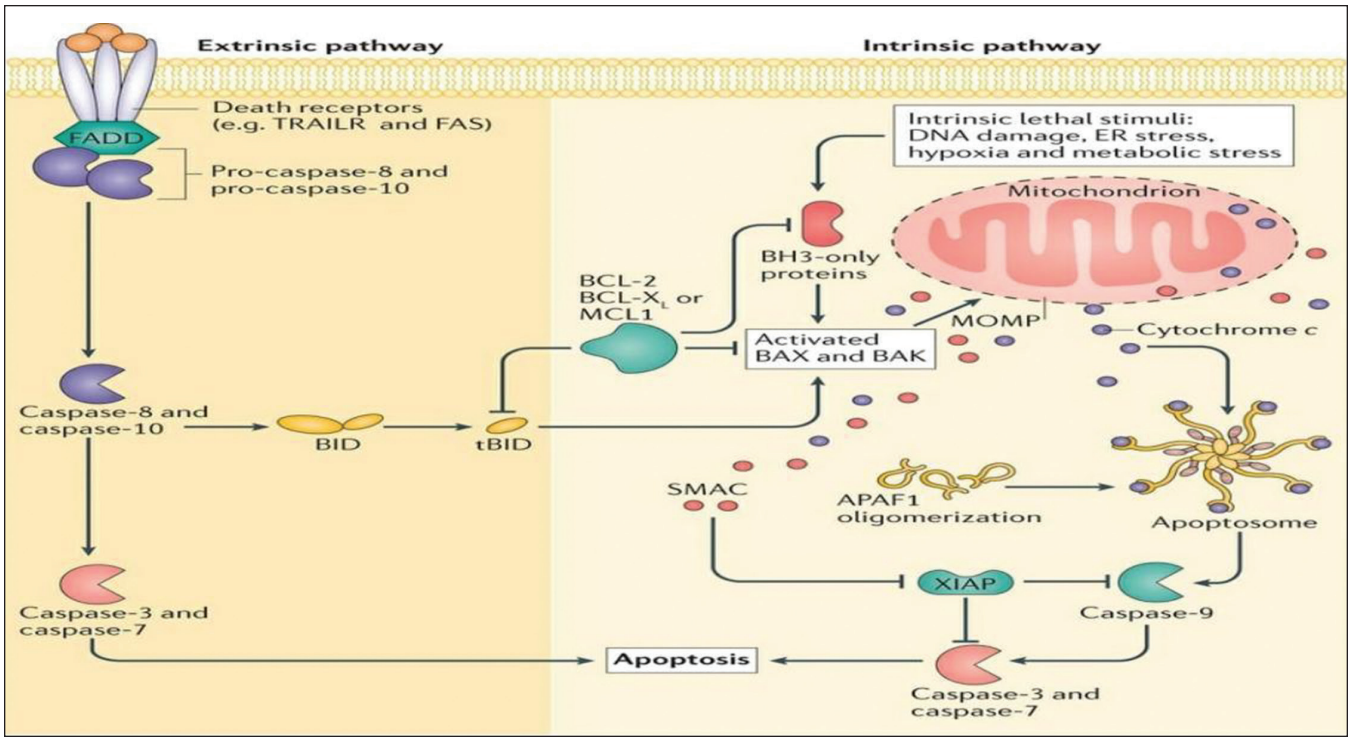
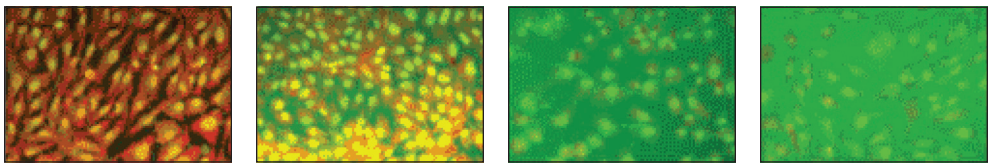


Figure 9. The Extrinsic and Intrinsic Pathways of Apoptosis. *(Eskandari and Eaves, 2022)*



Extract at IC_{50}	Apoptotic Index
2% DMSO	1.3 ± 0.07
CMLE-SS	23.3 ± 2.9
LEAF-SS	77.6 ± 4.7*
Doxorubicin	87.2 ± 5.6*

Figure 10. Fluorescence Micrograph of Apoptotic MCF-7 Breast Cancer Cells *(Green)* Exposed to **(A)** 2% DMSO, **(B)** Crude Methanol Leaf Extract of *S. samarengense* and its Ethyl Acetate Fraction **(B & C)** and Doxorubicin **(D)** at IC_{50} Concentrations.
*(*p < 0.001 vs control)*

antiproliferative effects of flavonoids in MCF-7 cells at 50 mM was mediated by selectively blocking estrogen receptor to suggest that adjacent hydroxyl groups in the molecules are essential for binding to this receptor.[21] The antiproliferative effects of genistein, another flavonoid, is mediated by similar mechanism.[22] *In silico* molecular docking experiments show the binding of the hydroxyl groups of flavonoids via hydrogen bonding with various amino acids in the active binding site of the estrogen receptor, as shown in Figure 11.[23]

A significant finding of this study is the non-cytotoxicity of CMLE-SS and LEAF-SS in non-tumorigenic breast epithelial cell lines. It is possible that bioactive substances contained therein can bind to target cellular receptors that expresses biomarkers of metastasis in MCF-7 cells without affecting normally-dividing cells. This selective cytotoxicity translates well to lesser adverse effects commonly associated with chemotherapeutic drugs. Fulvestrant, a selective estrogen receptor destabilizer, was found to be

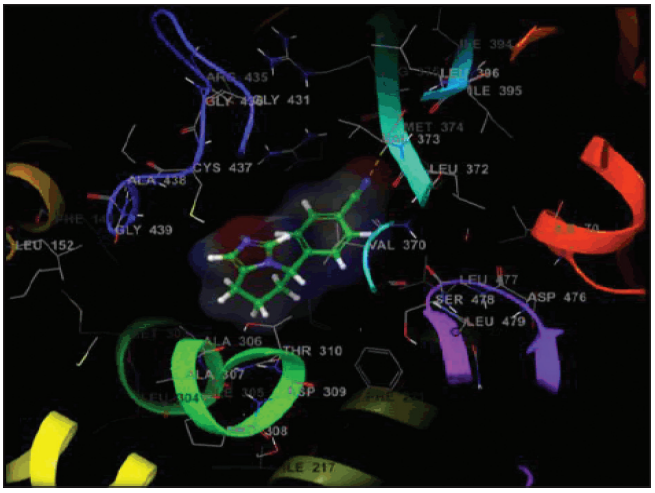


Figure 11. Interaction of a Flavonoid in the Active Site of Estrogen A Receptor.

cytotoxic only to metastatic hormone receptor - positive breast cancer cells but not to healthy and normally-dividing epithelial breast cells.[24]

By far, tamoxifen and raloxifene are the only available selective estrogen receptor modulators used to treat breast cancer, particularly in post-menopausal women. Letrozole and anastrozole are reversible aromatase inhibitors that block the conversion of androgens to estrogens. Flavonoids have been known to inhibit aromatase in addition to their ability to modulate estrogen receptors in non-invasive breast cancer cells, particularly in MCF-7 which is a luminal type of cells that expresses both estrogen and progesterone receptors.[25] For breast cancer cells that expresses HER-2 proteins, monoclonal antibodies such as trastuzumab are ideal substrates that block these receptors. Interestingly, flavonoids such as naringenin have been implicated to block HER-2 receptors in MDA-MB-231 breast cancer cell lines.[26] For both MCF-7 and HER-2 cells, the flavonoid quercetin suppresses production of VEGFR-2 which are endothelial growth factors necessary for angiogenesis via various cellular signaling pathways.[27] Downregulation of mTOR/MAPK cell signaling pathways, suppression of TNF-α production, and topoisomerase inhibition are other mechanisms by which flavonoids can exert cytotoxic and apoptotic effects against breast cancer cells. In highly metastatic luminal types of breast cancer expressing cyclin-dependent kinases (CDK 4 and 6), palbociclib can inhibit these enzymes; so far, flavonoids with this mechanism have yet to be discovered.[28] Chalcone flavonoids from the hexane fractions of the crude methanol leaf extract of *S. samarangense* were reported to be cytotoxic against MCF-7 and the highly tumorigenic HER-2 positive SK-BR-3 breast cancer cell lines.[29] The abundance of flavonoids in the LEAF-SS was established in this study making this extract promising as a potential herbal medicine that can be used as an alternative treatment for estrogen-receptor positive breast cancers.

CONCLUSION

The flavonoid-rich ethyl acetate fraction of the crude methanol leaf extract of *S. samarangense*, a Philippine endemic plant, exhibits potential antitumor action against MCF-7 breast cancer cell lines. This antitumor action was mediated by cytotoxicity via apoptosis, DNA fragmentation and caspase-3 activation. This extract was non-proliferative when tested against normal human epithelial breast cell lines and was suggestive of its selective cytotoxicity and safety.

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Statement of Authorship

GQDG contributed in the conceptualization, design of the experiments, and data interpretation with thorough discussion; MSFSC contributed in the manuscript revision, English editing, and discussion of results; DMAB contributed in the statistical analysis and preliminary discussion; SDVM contributed in the screening for cytotoxic and apoptotic properties.

Author Disclosure

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