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HPLC Isolate from the Leaves of Kaffir Lime (*Citrus hystrix* DC) Exhibits Selective Cytotoxicity Against Human Breast Adenocarcinoma Cell Line, MCF-7

Fatima Mariz Almenario* and **Sonia D. Jacinto**

Institute of Biology, National Science Complex, Antonio Ma. Regidor St.
University of the Philippines Diliman, Quezon City 1101 Philippines

The increasing mortality and morbidity rate caused by breast cancer warrants the need to screen for compounds ideal for chemotherapeutic application. This study evaluated the cytotoxicity of *Citrus hystrix* leaves utilizing a bioactivity-guided fractionation scheme. Using various chromatographic techniques, the fractions were subjected to MTT [3-(4, 5-dimethylthiazol-2-yl)-2, diphenyltetrazo-lium bromide] assay to determine cytotoxicity and bioassays to confirm apoptosis as the mode of cell death. The results revealed that the crude extract was moderately cytotoxic against human breast adenocarcinoma (MCF-7) but not to other cancer cell lines tested. Solvent partitioning of the crude extract revealed that hexane partition exhibited the highest cytotoxicity (IC_{50}: 23 µg/mL) to MCF-7. Sequentially, vacuum liquid chromatography (VLC) of the hexane partition separated VLC 7 (IC_{50}: 17.2 µg/mL) with the highest percent yield. Hence, VLC 7 was subjected to gravity column chromatography (GCC), where GCC 7.4 exhibited promising cytotoxicity to MCF-7 (IC_{50}: 14.6 µg/mL). Selectivity index (SI) indicated that GCC 7.4 was highly selective against MCF-7 (SI > 2), relative to non-cancer cell lines NIH3T3, HDFn, and AA8. Interestingly, GCC 7.4 induced apoptosis as confirmed by several markers including phosphatidylserine (PS) membrane translocation, mitochondrial membrane depolarization, nuclear condensation, and DNA fragmentation. To further purify the components present in GCC 7.4, high-performance liquid chromatography (HPLC) was performed. HPLC 7.4.5 was active against MCF-7 (IC_{50}: 5.5 µg/mL) without being cytotoxic against the non-cancer cell line NIH3T3. Overall, the results demonstrated that *C. hystrix* leaves possess promising compounds that are highly selective and cytotoxic against MCF-7 by inducing apoptosis.

Keywords: apoptosis, cancer, *Citrus hystrix*, cytotoxicity, MCF-7, plant natural products

INTRODUCTION

Cancer is a major health concern due to its rapidly growing incidence and mortality rates worldwide. It is considered the second leading cause of death, next to cardiovascular diseases (Bray *et al.* 2018; Siegel *et al*. 2020). The recent data document 9.6 M cancer-related deaths and 18.1 M new cancer cases in 2018 (Bray *et al.* 2018). Among the top cancer cases, breast cancer is the most prevalent cancer among females and is the principal cause of cancer death in women worldwide (Siegel *et al.* 2020). Despite recent advances in medicine and technology, this high mortality rate indicates the complexity of breast cancer and the limitations of current cancer therapies (Pucci *et al.* 2019). Nonetheless, chemotherapy remains to be one of the most extensively and commonly used approaches for the management and treatment of cancer (Sak 2012). Developing an anticancer agent requires low organ toxicity, high specificity to target cells, and less adverse effects (Sak 2012) – all of which are critical factors that

*Corresponding Author: fatzalmenario@gmail.com

limit the usefulness of today's anticancer drugs. This then warrants the need to screen for novel compounds with anticancer properties ideal for future chemotherapeutic application.

Plant natural products are major sources for the development of anticancer drugs. In contrast to some commercially available anticancer agents which pose low selectivity and greater side effects (Desmain and Vaishnav 2011), plant-derived compounds exhibit greater therapeutic efficiency, promote fewer undesirable side effects, and induce less mutagenicity in normal cells (Sak 2012; Tunjung *et al.* 2015). In fact, several known anticancer drugs have been isolated and developed from plants.

The Philippines has potentially rich plant biodiversity, which explains why studies on plant natural products and their potential bioactivities are widely explored and increasingly studied over the past years. *Citrus hystrix*, commonly known as kaffir lime, is a Rutaceae plant native to the Philippines and other Southeast Asian countries. This plant is widely distributed and cultivated in many parts of the country. The leaves of *C. hystrix* are used mainly as an ingredient in Asian cuisines and have been traditionally used as a natural alternative for scurvy and, thus, maintain healthy gums (Anuchapreeda *et al*. 2020a). Several reports have demonstrated that *C. hystrix* leaves and fruit peels exhibit a broad array of biological activities including antibacterial, anti-inflammatory, antioxidant, antifungal, hepatoprotective, and cardioprotective properties (Agouillal *et al*. 2017). This wide spectrum of biological activities suggests that *C. hystrix* contains a plethora of secondary metabolites proving ideal for the screening of potential anticancer compounds. In fact, bioactive compounds isolated from various citrus plants have exerted notable anticancer properties. For example, limonoids from citrus fruits exhibit cytotoxicity against neoblastoma and colon cancer cells (Poulose *et al*. 2006). The volatile oil from *C. aurantifolia*, which contains D-limonene and D-hydrocarvone, demonstrated cytotoxic effects against colon cancer cells (Patil *et al*. 2009), while essential oils from *C. hystrix* leaves and peels were found to inhibit cervical and leukemic cell lines (Manosroi *et al*. 2006).

Previous studies have reported the cytotoxic activity of *C. hystrix* leaves. For example, Tunjung *et al*. (2015) demonstrated that ethyl acetate (IC_{50}: 14.1–40.7 µg/mL) and chloroform extract (IC_{50}: 6.4–18.9 µg/mL) of this plant exhibit cytotoxicity against HeLa and neuroblastoma cell lines. In addition, ethanolic and ethyl acetate fraction proved to be cytotoxic against HeLa cells with IC_{50} of 82 mg/mL and 57.8 mg/mL, respectively (Wijayanti *et al*. 2015). Moreover, the ethyl acetate fraction of *C. hystrix* consistently demonstrated activity against leukemic cell lines with IC_{50} ranging from 19–35 µg/mL (Chueahongthong *et al.* 2011). Despite this, little is known regarding the activity and selectivity of *C. hsytrix* leaves on human breast cancer cell line MCF-7. Also, the majority of cytotoxicity studies of *C. hystrix* leaves only tested crude and solvent partitions. Hence, further purification techniques should be performed to screen its anticancer activity necessary for potential drug development. Using HPLC, this study tested a semi-pure isolate from *C. hystrix* leaves, which proved to be selectively inhibitory against MCF-7.

Overall, this study aims to 1) separate bioactive compound/s using a bioassay-guided fractionation, 2) determine the cytotoxicity and selectivity of active fractions against cancer and non-cancer cell line using MTT assay, and 3) investigate the mechanism of cell death induced by the active fraction by using apoptotic markers.

METHODS

Plant Collection and Sample Preparation

Mature, healthy leaves of *C.hystrix* were collected during the summer season from Mt. Lamao, Bataan (14º 30' 46" North, 120º 36' 26" East), Philippines. A voucher specimen was deposited (Accession No. 21415) at the Jose Vera Santos Memorial Herbarium, Institute of Biology, University of the Philippines Diliman, Quezon City. The rest of the collection was then processed for extraction. Briefly, leaves were washed, oven-dried at 35 ºC, powdered, and macerated in distilled methanol (RCI Labscan Ltd., Thailand) for at least 72 h. Then, the extract was filtered and concentrated using a rotary evaporator (Heidolph, Germany), air-dried at room temperature, and stored at 4 ºC for future use. The extraction process was repeated three times to obtain 35 g of crude extract from 300 g of macerated plant sample.

Bioactivity-guided Fractionation

As a means of producing active isolates, solvent partitioning, and chromatographic techniques were performed sequentially. The partitions and fractions collected from each step were subjected to MTT assay to determine their cytotoxic activity, which served as a basis for the selection of the fraction/s for the next purification stage.

Solvent partitioning. Approximately 30 g of crude extract was partitioned exhaustively using three solvents of increasing polarity, *i.e.* hexane (RCI Labscan Ltd., Thailand), ethyl acetate (RCI Labscan Ltd., Thailand), and water. The crude extract was dissolved in 1:1 water to hexane, sonicated to obtain a homogenous mixture. Using a separatory funnel, the solution was allowed to settle and separate for at least 4 h. After settling time, the upper hexane portion of the extract was collected. The partition

was concentrated using a rotary evaporator and the process was repeated until all soluble hexane components were extracted. Similar procedures were followed for ethyl acetate and aqueous solvents used sequentially. The aqueous partition was subjected to lyophilization (Alpha 1-2 LDplus Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Finally, the concentrated partitions were tested for cytotoxicity using the MTT assay. Because hexane partition was the most active, it was subjected to the next purification step. Approximately 30 g of crude extract yielded 5 g of the active hexane partition.

VLC. About 5 g of hexane partition was added with the same amount of 60G (Merck KGaA, Darmstadt, Cat # 1.077234.1000) silica gel, dissolved in hexane, and concentrated using a rotary evaporator. The extract was loaded onto a vacuum dry-packed 60G silica gel column in a Buchner funnel. The amount of the silica gel used was 50x more than the weight of the sample. The process used 200 mL of 100% hexane to hexane-ethyl acetate gradients at 10% increments, ending with 100% absolute ethanol (RCI Labscan Ltd., Thailand). Each eluent was collected after all the solvent has passed through the column. The collected VLC fractions were concentrated in a rotary evaporator and bioactivity was tested through MTT assay. Among the active VLC fractions (VLC 6-12), VLC 7 produced the highest yield of 576 mg from 5 g of hexane partition (yield: 12%) and was, thus, selected for the next purification stage.

GCC. In this method, 60G (Merck KGaA, Darmstadt, Cat # 1.077231.1000) silica gel was packed following the slurry method. The amount of the silica gel used was 100x the amount of the sample. Approximately 200–250 mg of VLC 7 was dissolved in its respective solvent system and was carefully loaded onto the column. The eluting solvent was changed in order of increasing polarity from 6:4 hexane-ethyl acetate gradient and ending at 100% absolute ethanol. Each band was collected separately and concentrated using a rotary evaporator, then tested for their respective cytotoxicity. Among the active GCC fractions (GCC 7.2–7.4), GCC 7.4 produced a significant yield of 85.3 mg from 200 mg of VLC 7 (yield: 43%). This served as the basis for GCC 7.4 to be purified using HPLC.

HPLC. GCC 7.4 was subjected to HPLC with 50:50 acetonitrile-methanol solvents in an isocratic elution using the C-18 reverse phase column (Inertsil, 4.6 x 250 mm, Japan, Cat # 5020-01732). Briefly, 100 ppm of GCC 7.4 was profiled using 1:1 acetonitrile-methanol. Using the selected HPLC chromatogram, each peak was collected from a previously prepared 1000 ppm of the sample. Collected HPLC isolate/s were evaluated for cytotoxicity against cancer and non-cancer cell lines.

Cell Culture and Maintenance

The cell lines used in the study were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured at the Mammalian Cell Culture Laboratory, University of the Philippines Diliman. All cell lines were incubated at 37 ºC and 5% CO_2 with 95% humidity.

The following human cancer cell lines were used: breast adenocarcinoma MCF-7 and MDA-MB-231, lung adenocarcinoma A549, and colorectal carcinoma HCT-116. Each cell line was grown in the recommended media formulations. Unless otherwise stated, all products were supplied by Life Technologies (Gibco, NY, USA). MCF-7 was maintained using 88% minimum essential medium supplemented with 10% fetal bovine serum (FBS), 1% insulin-transferrin-selenium-sodium-pyruvate, and 1% antibiotic-antimycotic (AA). A549 was grown using 89% F-12 (1x) nutrient mixture (Ham), 10% FBS, 1% sodium bicarbonate, and 10 µL of 10 mg/mL gentamicin. HCT-116 was maintained using 88.5% McCoy's 5A medium (1x), 10% FBS, 1.5% sodium bicarbonate, and 10 µL of 10 mg/mL gentamicin. Finally, MDA-MB-231 was grown using a medium containing 89% Dulbecco's modified essential medium or DMEM (1x), 10% FBS, and 1% AA.

The non-cancer cell lines used include mouse embryonic fibroblast NIH3T3, Chinese hamster ovarian AA8, and human dermal fibroblast HDFn. NIH3T3 was grown using 90% DMEM (1x), 10% FBS, and 10 µL of 10 mg/mL gentamicin. AA8 was maintained using media containing 90% RPMI medium, 10% FBS, and 10 µL of 10 mg/mL gentamicin. HDFn was grown using medium containing 90% DMEM, 10% FBS, and 0.01% of 10 mg/mL gentamicin.

Cell Viability Assay

The cytotoxic activity of the fractions was evaluated using the MTT assay. Details of the MTT assay were adopted from Mosmann (1983) with minor modifications. Briefly, cells were seeded in a 96-well plate at a density of 6 x 10^4 cells/mL (MCF-7) and 4 x 10^4 cells/mL (for other cell lines) and incubated at 37 ºC for 24 h. In a master dilution plate (MDP), 10 mg/mL of crude extract or fractions were obtained and serially diluted two-fold. For 72 h, the plated cells were treated with 10 µL of eight serially-diluted concentrations from the MDP, making a final concentration of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.5625 µg/mL, and 0.78125 µg/mL. Then, the treatment was terminated by adding 20 µL of MTT dye [5 mg/mL phosphate-buffered saline solution (PBS)] per well with 4-h incubation. Insoluble formazan crystals were dissolved using 150 µL dimethyl sulfoxide or DMSO (RCI Labscan Ltd., Thailand). The colorimetric reaction was measured using LeDetect microplate reader (Labexim, Eu) at 570 nm. Absorbance readings, expressed as percent (%) inhibition,

were used to compute for the IC_{50} value using Graphpad Prism 6.01. The IC_{50} values were used to calculate for the SI obtained by dividing the IC_{50} of the non-cancer cell line by the IC_{50} the cancer cell line. The anticancer drug doxorubicin (Hospira, Australia) served as the positive control agent while DMSO, the vehicle solvent, was used as the negative control. MTT assay was performed three times for each test sample.

Assays Determining Induction of Apoptosis

Among the active GCC fractions, GCC 7.4 was selected to determine its ability to induce apoptosis. This selection was based on the yield, cytotoxicity, and selectivity to MCF-7. Several marker assays were performed to detect the occurrence of apoptosis.

PS membrane translocation by annexin V/PI staining. The external translocation of PS from the inner to the outer leaflet of the plasma membrane is one of the early markers of apoptosis. To observe this event, annexin V/PI (Invitrogen, Life Technologies, New York, USA) assay was performed. MCF-7 was seeded at a density of 1 x 10^5 cells/ well in a six-well plate and incubated for 24 h then treated with paclitaxel (positive control) to a final concentration of 5 µg/mL, DMSO (negative control), and 15 µg/mL of GCC 7.4 (IC_{50}) for 48 h. Cells were then harvested using 0.25% trypsin-EDTA, centrifuged, and resuspended in annexin-binding buffer. Approximately 1 x 10^5 cells per treatment were incubated with 5 µL FITC annexin V and 1 µL of 100 µg/mL propidium iodide (PI) for 15 min at room temperature. Stained cells were resuspended, washed, centrifuged, and deposited into a 96-well plate. The cells were counterstained using 2:1000 Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloridetrihydrate) (Cellstain, Dojindo) in PBS and incubated for 30 min at 37 ºC. The counterstained cells were washed, centrifuged, and imaged under a fluorescence microscope (Carl Zeiss, Axio VertA.1) using FITC, Cy-3, and DAPI (4′,6-diamidino-2-phenylindole) bandpass emission filters.

Mitochondrial membrane depolarization by JC-1 assay. In this experiment, JC-1 assay (MitoProbe, Life Technologies, New York, USA) was performed to monitor changes in mitochondrial potential, which characterize initial phases of the intrinsic apoptosis pathway. Briefly, MCF-7 were seeded at a density of 6 x 10^4 cells/mL in a 96-well plate and incubated at 37 ºC for 24 h. Then, cells were treated with carbonyl cyanide m-chlorophenylhydrazone or CCCP (positive control) to a final concentration of 50 µM, DMSO, or the IC_{50} of GCC 7.4 for 48 h. Subsequently, treated cells were stained with 2 µM/well of JC-1 fluorescence dye and incubated for 15–30 min at 37 ºC, in the dark. The stained cells were centrifuged, washed, and imaged using a fluorescence microscope under FITC and Cy3 filters. Finally, absorbance was read at 535/595 nm and 485/535 nm, with a Varioskan™ LUX (Thermo Fisher Scientific, Massachusetts, USA).

Nuclear morphological changes by Hoechst staining. To observe changes in nuclear morphology in treated cells, Hoechst 33342 staining was performed. Briefly, MCF-7 were seeded at a density of 6 x 10^4 cells/mL in a 96-well plate and incubated for 24 h at 37 ºC. Then, cells were treated with paclitaxel (positive control) at a final concentration of 5 µg/mL, DMSO, or the IC_{50} of GCC 7.4 for 48 h. Cells were then stained with 2:1000 Hoechst 33342 solution and incubated for 30 min at 37 ºC then imaged under bright field and fluorescence microscope using DAPI filter.

DNA fragmentation by TUNEL assay. To determine the presence of DNA strand breaks on MCF 7-treated cells, TUNEL or terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling assay (DeadEndFluorometric TUNEL System, Promega, USA) was performed. Briefly, cells were seeded at a density of 6 x 10^4 cells/ml in a 96-well plate, incubated for 24 h at 37 ºC. Cells were then treated with paclitaxel (positive control) at a final concentration of 5 µg/mL, DMSO, or the IC_{50} of GCC 7.4, for 72 h. Thereafter, cells were fixed with 4% paraformaldehyde for 25 min, permeabilized with 0.25% Triton X-100 for 10 min, and incubated with TdT reaction cocktail for 1 h. The cells were stained with 2x SSC for 15 min, washed and counterstained with Hoechst 33342 then imaged under fluorescence using FITC and DAPI filters.

Statistical Analyses

MTT cytotoxic test and apoptotic assays were performed in three independent trials with two replicates per trial. Depending on the normality test results, the one-way analysis of variance or Kruskal-Wallis test was used to compare means between different treatment groups ($\alpha = 0.05$). To determine significance between and within treatments, Tukey's *post hoc* or Dunn's test was used. Statistical analyses were performed using Graph Pad 6.0.

RESULTS

C. hystrix Leaf Extracts Exhibited Cytotoxicity to MCF-7

The cytotoxicity of *C. hystrix* against selected cancer cell lines was measured in terms of the IC_{50} value (µg/mL) or the concentration of the test extract that will inhibit 50% of the cell viability. Based on the set value by the American National Cancer Institute, an extract with an

IC_{50} value of 30 µg/mL or less is considered active and is worthy of further purification steps (Suffness and Pezzuto 1990). As shown, *C. hystrix* crude extract was nearly cytotoxic against MCF-7 with an IC_{50} of 33 µg/mL. However, the crude extract was found to be inactive against human colorectal cancer (HCT-116) and human lung adenocarcinoma (A549) cell lines with an IC_{50} value of 100 µg/mL (Figure 1A). Although the obtained IC_{50} value for MCF-7 was a little above the standard toxic value, the crude extract was subjected to various purification steps because of its selectivity to MCF-7.

The cytotoxicity results of the solvent partitions revealed that hexane fraction is relatively more cytotoxic (IC_{50}: 23 µg/mL) against MCF-7 compared to ethyl acetate and aqueous partitions (Figure 1B). This indicates that non-polar compounds constitute the majority of the active components present in the extract. With this, the hexane partition was selected for further fractionation using VLC.

The results yielded 11 distinct fractions, seven of which are active (VLC 6 to VLC 12) with an IC_{50} of less than 30 µg/mL (Figure 1C). It must be noted that VLC 13 did not produce significant yield to be tested for their cytotoxicity. Because further fractionation steps require sufficient yield, VLC 7 – which generated the highest yield of 12% (IC_{50}: 17.2 µg/mL) – was chosen for the next purification stage.

The GCC of VLC 7 separated six fractions, three of which were cytotoxic against MCF-7 (Figure 1D). Although GCC 7.3 exhibited the most potent cytotoxicity (IC_{50}: 12 µg/mL), it produced insufficient yield (24%), along with the rest of the active GCC fractions. Hence, GCC 7.4 was selected for the final purification step on the basis of its activity (IC_{50}: 14.6 µg/mL) and percent yield (43%). Using HPLC, GCC 7.4 yielded five HPLC isolates, with HPLC 7.4.5 showing cytotoxicity against MCF-7 (IC_{50} 5.5 µg/mL) while the rest of the isolates demonstrated no activity at all (Figure 1E).

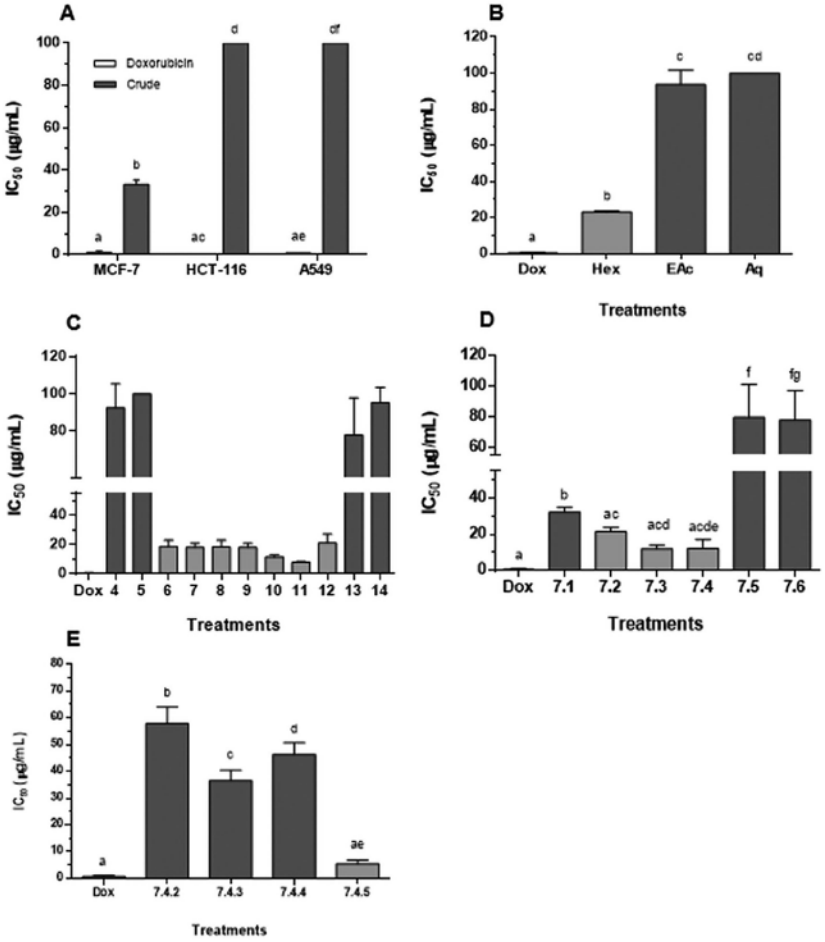

Figure 1. Cytotoxicity of *C. hystrix* leaf extracts against selected cancer cell lines following 72 h treatment. The bars represent mean IC_{50} (± SD) values (µg/mL) of positive control doxorubicin and A) *C. hystrix* crude extract, B) solvent partitions, C) VLC fractions, D) GCC fractions, and E) HPLC fractions. Bars with the same letter are not significantly different ($\alpha = 0.05$).

C. hystrix GCC 7.4 is Selectively Inhibitory Against MCF-7

To test the range of cytotoxicity to other cancer cell lines, GCC 7.4 was tested against the sturdy triple-negative breast cancer (MDA-MB-231) and lung adenocarcinoma (A549) cell lines. The results revealed that GCC 7.4 was strictly cytotoxic to MCF-7 but not to other cancer cell lines (Figure 2A). Interestingly, GCC 7.4 did not exhibit cytotoxicity to any of the non-cancer cell lines tested (Figure 2B). SI was used to measure the selectivity of the extract on cancer cell line relative to the non-cancer cell line. The results demonstrated that GCC 7.4 has high SI (> 2) to MCF-7 (Figure 2C). Similarly, HPLC 7.4.5 was found inactive against the non-cancer cell line NIH3T3 and, therefore, proved to be highly selective on MCF 7 (SI: 17.2).

C. hystrix GCC 7.4 Induces Apoptosis on MCF-7

To identify whether GCC 7.4 effectively induces apoptosis on MCF-7, several marked events were observed using apoptosis assays. Annexin/PI staining was performed to determine translocated PS, a biomarker for early apoptotic cells. The results revealed that GCC 7.4 was able to induce PS translocation, as evidenced by the presence of annexin-positive cells (Figure 3). The observed effect in GCC 7.4-treated cells was comparable with paclitaxel, the positive control agent used. Furthermore, the presence of numerous PI-positive cells on GCC 7.4 signifies the presence of cells that underwent late apoptosis.

Mitochondrial membrane depolarization is a significant event that indicates the occurrence of intrinsic apoptosis. To examine whether this pathway is activated on MCF-7 treated cells, JC-1 assay was performed. As shown, cells treated with GCC 7.4 exhibited intensely-stained green fluorescence comparable to treatment with CCCP, a mitochondrial membrane disruptor used as the positive control agent (Figure 4A). This strongly indicates compromised mitochondrial membrane and dysfunction. Conversely, DMSO-treated cells possessed an active mitochondrial membrane, as evidenced by predominantly red JC-1 aggregates. This observation was supported by the spectrophotometry analysis expressed as red to green fluorescence ratio. As demonstrated, GCC 7.4-treated cells possessed low red to green signal ratio with no significant difference ($p > 0.05$) to CCCP-treated cells (Figure 4B).

Apoptotic cells typically exhibit malformations on nuclear morphology, an event that can be observed using Hoechst 33342 staining. As shown (Figure 5), normal cells have round and evenly-stained nuclei, as observed in DMSO-treated cells. However, MCF-7 cells treated with GCC 7.4 and positive control paclitaxel exhibited intensely-stained nuclei, reduction of nuclear size, and less defined borders. Moreover, using phase-contrast microscopy, cells treated with DMSO displayed an attached monolayer of cells with polygonal contours, a morphology typical of an epithelial MCF-7 cell line. On the other hand, GCC 7.4 and paclitaxel treatments both resulted in clumped, highly disorganized cells with detached morphology.

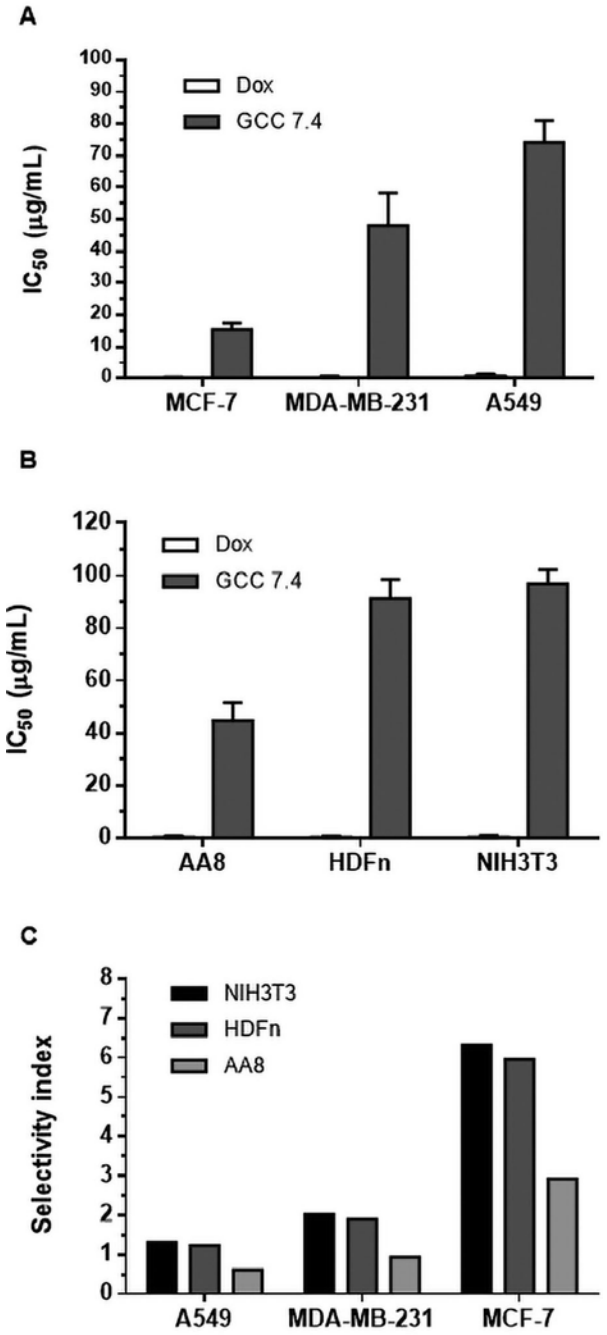

Figure 2. Cytotoxicity of GCC 7.4 and positive control doxorubicin following 72 h treatment. The bars represent mean IC_{50} (± SD) values (µg/mL) from MTT assay against (A) cancer and (B) non-cancer cell lines. SI of GCC 7.4 on various cancer cell lines is shown with high selectivity to MCF-7 (C).

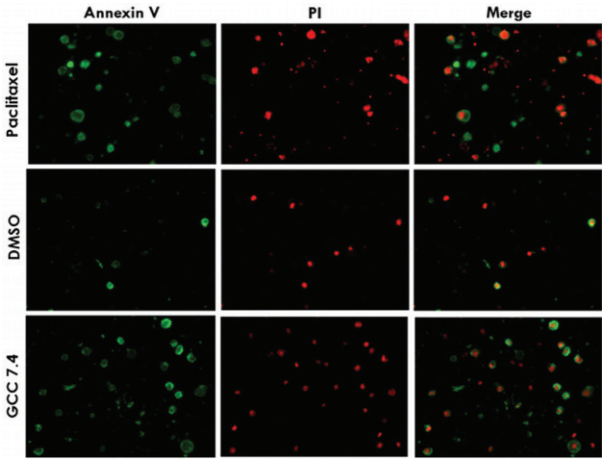

Figure 3. Detection of PS membrane translocation on MCF-7 following 48 h Annexin V/PI staining. The images indicate apoptotic cells (green) and late apoptotic cells (red). Paclitaxel, a known anticancer agent, was used as the positive control.

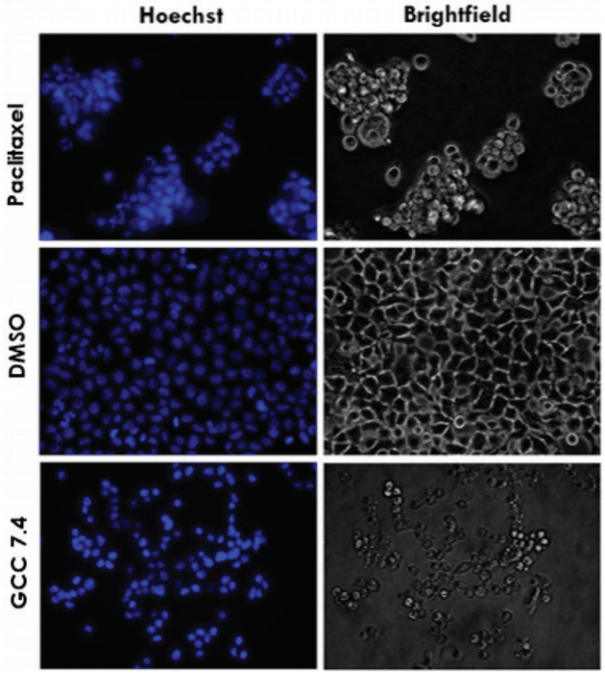

Figure 5. Detection of nuclear morphological changes on MCF-7 following 48 h Hoechst 33342 staining. Cells were stained with Hoechst 3342 to visualize nuclear morphology (left panel) and cell surface morphology viewed under bright field (right panel).

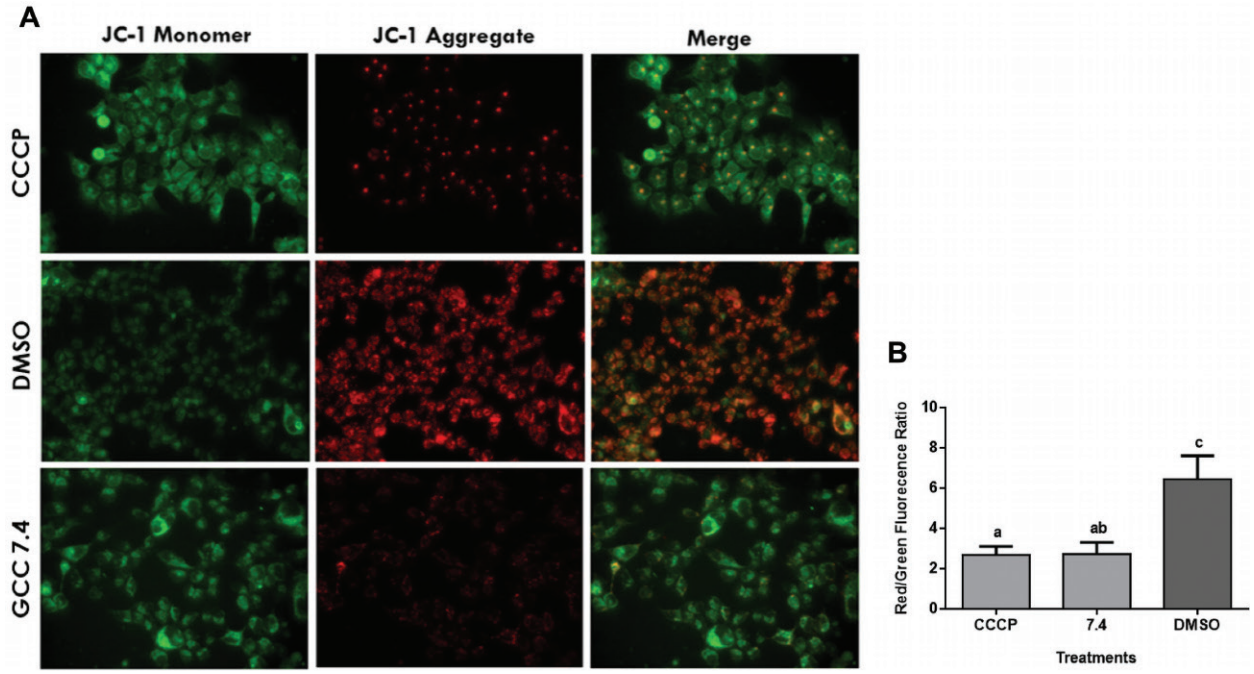

Figure 4. Detection of mitochondrial membrane depolarization of MCF-7 following 48 h JC-1 staining (A). Mitochondrial membrane potential in treated cells is expressed as red to green fluorescence ratio (B). CCCP, a mitochondrial membrane disruptor, was used as the positive control. Bars with the same letter are not significantly different ($\alpha = 0.05$).

DNA fragmentation is one of the characteristic hallmarks of apoptosis, which can be detected by performing a method known as the TUNEL assay. The results show that cells treated with GCC 7.4 and paclitaxel, a known anticancer agent, induced DNA fragmentation following 72 h of treatment (Figure 6). This is revealed by the presence of TUNEL-positive cells. The observation was consistent for three trials, suggesting that GCC 7.4 treatment effectively causes DNA fragmentation in the MCF-7 breast cancer cell line.

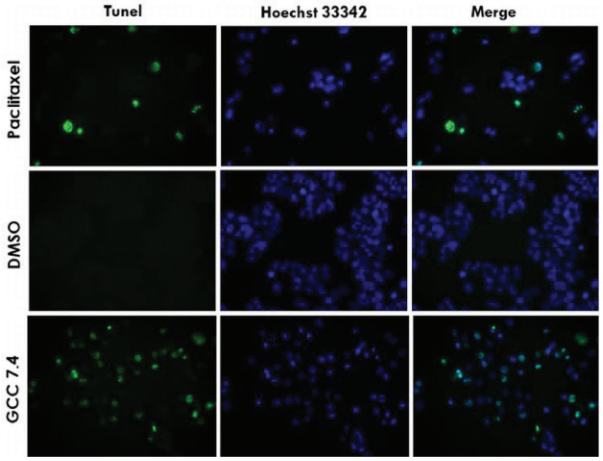

Figure 6. Detection of DNA fragmentation in MCF-7 following 72 h treatment with TUNEL staining. The cells were counterstained with Hoechst 33342.

DISCUSSION

This study reported that *C. hystrix* crude extract contains compounds that are selectively inhibitory against MCF-7, which is why several purification steps were performed to purify the cytotoxic components present. The results revealed that hexane partition contains a cocktail of cytotoxic components, suggesting that non-polar compounds constitute the major bioactive components and, thus, making them likely candidates for isolation of promising anticancer compounds.

One of the interesting findings in this study is that GCC 7.4 was particularly selective only to the epithelial type breast cancer cell line MCF-7 but not to the highly metastatic and invasive breast cancer cell line MDA-MB-231, nor the sturdy lung adenocarcinoma A549. This observed differential susceptibility of the cancer cell lines toward the extract can be attributed to the differences in their origin, morphology, and genome (Tunjung *et al.* 2015). The MCF-7 cell line is a luminal type A subtype that overexpresses estrogen/progesterone receptors on its membrane and is minimally invasive (So *et al.* 1997). On the other hand, the MDA-MB-231 cell line is a triple-negative basal subtype (ER–/PR–HER2–) that is highly aggressive and metastatic (Moses and Edwards 2016). Because MCF-7 mainly depends on estrogenic receptor signaling pathway for growth and survival, it may be speculated that the active compound/s present in GCC 7.4 possess anti-estrogenic properties, which specifically target estrogen-receptor binding critical for the transcription of genes (c-myc, cyclins D) necessary for breast cancer proliferation, differentiation, and overall survival (Huang *et al.* 2012). In addition, it may be likely that the active compound could block the synthesis of estradiol, particularly by interfering with post-translational modifications (Kiyama 2017). A recent study by Anuchapreeda *et al.* (2020a) isolated agrostophillinol with cytotoxicity against leukemic cell lines EoL and HL60. A separate study by Anuchapreeda *et al.* (2020b) isolated phytol and lupeol from the Fraction 9 of the hexane partition. These compounds demonstrated cytotoxicity and antiproliferative activity on leukemic cell line K562. Although it may be speculated that these identified putative compounds may be responsible for the observed cytotoxicity and apoptosis-inducing ability of GCC 7.4 against MCF-7, it may also be highly likely that the bioactive compounds present are different because the purification method they used was different from the methods we performed. It must also be noted that the type and amount of secondary metabolites are influenced by several factors – including geographical location, environmental condition, season of collection, age of the plant, and extraction methods (Ramakrishna and Ravishankar 2011).

The results revealed that GCC 7.4 did not exhibit cytotoxicity against any of the non-cancer cell lines, as evidenced by its high SI (17.2). According to Al-Qubaisi *et al.* (2011), an extract possesses selective cytotoxicity if its SI value is greater than two. This then further validates that at the level of GCC fractionation, 7.4 can already be potentially studied for isolation and development of chemotherapeutic drugs specifically against human breast cancer cell line MCF-7, an E/R+ cell line, or a similar type of breast cancer.

Moreover, this study confirmed that GCC 7.4 induced apoptosis on MCF-7. Apoptosis, unlike necrosis, is a type of cell death that does not release its cellular contents and other materials to surrounding cells, which may lead to an immune and inflammatory response. These apoptotic cells form apoptotic bodies, which are finally cleared through phagocytic uptake of macrophages – a rather silent way of eliminating aberrant cells (Hanahan and Weinberg 2011). Cancer cells, however, have developed apoptosis-resistance strategies to bypass this mode of cell death and, therefore, any compound that induces apoptosis is an ideal and a promising anticancer agent.

The translocation of PS from the inside, where it is normally found, to the outer membrane is one of the early

events of apoptosis. The exposure of PS unto the surface of apoptotic cells signals the recruitment of professional and non-professional phagocytes to bind and take action (Brouckaert *et al.* 2004). In the study, annexin V/PI staining was performed, which utilizes the principle that annexin V – a calcium-dependent protein has a high affinity for PS – while PI is a DNA-binding dye that permeates cells with compromised cell membrane, thereby marking early and late apoptotic or necrotic cells (Vermes *et al.* 1995). The results showed that GCC 7.4 treatment induced early and late apoptosis, as evidenced by the presence of annexin- and PI-positive cells. This exposure of PS unto the membrane surface may possibly signify that upstream apoptotic events have already been activated. This is because PS translocation can be mediated by the activation of caspases 3 and 7, which in turn activates XRP8, a lipid scramblase that translocates PS to the outer membrane surface (Peng *et al.* 2001).

Mitochondrial membrane depolarization is an irreversible event that occurs in the early stages of apoptosis. This activates the cascading events leading towards the intrinsic apoptosis pathway (Ding *et al*. 2012). This study used the JC-1 probe – a green, fluorescent, lipophilic dye that forms red aggregate inside active mitochondria. However, apoptotic cells with inactive mitochondria and, hence, the depolarized membrane cannot form red JC-1 complexes – allowing JC-1 monomers to remain in the cytoplasm – in its green monomeric state (Perelman *et al*. 2012). In the experiment, GCC 7.4 treatment was able to induce membrane depolarization on MCF-7 treated cells as indicated by its low red to green fluorescence ratio. This result supports the cell viability test obtained from the MTT assay of GCC 7.4-treated cells. Cells with dysfunctional mitochondria and, hence, a compromised mitochondrial activity cannot convert MTT into formazan products, suggesting that the lethal effect of the extract was probably aided by the initiation of mitochondrial membrane depolarization (Acebedo and Jacinto 2014). Additionally, this result supports the possibility that the release of PS to the membrane surface as detected by annexin staining, consequently resulted from the destabilization of the mitochondrial membrane, which activates caspases that act on scramblase proteins with PS-translocating activities (Peng *et al.* 2001).

One of the indicators of apoptosis is nuclear condensation caused by DNA fragmentation. The results showed that GCC 7.4-treated cells exhibited condensed nuclei, reduced nuclear size, and deformed nuclei with irregular borders. These nuclear morphological alterations can be attributed to either apoptosis or necrosis. Although it cannot be directly concluded that the effect was due to apoptosis, it was observed that the morphology of cells treated with GCC 7.4 does not display necrotic-like characteristics. Apoptotic cells are generally characterized by cell shrinkage and rounding of cells that eventually form apoptotic bodies, while necrotic cells exhibit the massive formation of surface evaginations or blebbing, swelling of cell and organelle, rupture of the plasma membrane, and the release of cytosolic materials. Here, GCC 7.4-treated cells displayed apoptotic-like features like the rounding of cells and shrinking of cells while retaining the integrity of its membrane. In contrast, it will be interesting to note that the morphology of paclitaxel-treated cells tends to appear swollen, clumped, and with distinct zeiotic blebs.

During the late stages of apoptosis, cells undergo extensive DNA fragmentation. The integrity of the DNA is crucial to cell survival and, hence, cells with compromised DNA undergo cell death. The results indicate that GCC 7.4 effectively caused DNA degradation, as shown by the presence of TUNEL-positive cells. This finding supports the activation of the intrinsic apoptosis pathway, which starts from mitochondrial membrane depolarization leading to the activation of caspases that cleave DNA strands. It can also be observed that paclitaxel, a chemotherapeutic drug, showed minimal effect on causing DNA breaks as compared to the effect induced by GCC 7.4 treatment.

This study was able to obtain a semi-pure HPLC 7.4.5 with potent cytotoxicity and selective activity against MCF-7. However, during the final course of the experiment and after 4 mo of storage at 4 ºC in the dark, the active HPLC 7.4.5 was found to have lost its cytotoxicity and, thus, unstable when stored at these conditions and duration. Hence, apoptotic assays and compound identification tests could not be performed on the isolate. It is, therefore, suggested to store the active HPLC isolate at ultra-low temperature conditions, which may retain its bioactivity and lengthen its shelf life.

Nevertheless, this study established notable findings such as good selectivity and apoptotic-inducing ability of the semi-pure GCC 7.4 on the human breast adenocarcinoma cell line. This, therefore, confirmed that at the level of GCC fractionation, fraction 7.4 contains active components that exhibit properties ideal of an anticancer agent. Also, it can be observed that the cytotoxic activity of *C. hystrix* improved from 33 μg/mL in the crude extract down to 5 μg/mL in the HPLC isolate, with an incremental change in toxicity as the purification steps progressed. This is in contrast to other plant purification studies where the activity of the extracts was typically lost as they were chemically purified along the way. Most importantly, the semi-pure HPLC fraction should be subjected to chemical characterization and structure elucidation tests to identify the compound responsible for the cytotoxic activity to MCF-7.

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