

Anti-breast cancer potential of frullanolide from *Grangea maderaspatana* plant by inducing apoptosis

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Abstract. Breast cancer is the leading cause of female mortality worldwide. Although there are several modern treatments for breast cancer, there is a high rate of recurrence for the majority of treatments; therefore, the search for effective anticancer agents continues. The present study aimed to investigate the anti-breast cancer potential of frullanolide, a compound which is isolated and purified from the *Grangea maderaspatana* plant, for selected human breast cancer cell lines (MCF-7, MDA-MB-468 and MDA-MB-231). The MTT assay was used to assess cytotoxic activity in breast cancer cell lines of treatment with frullanolide at 1.25, 2.5, 5.0, 10.0 and 20.0 $\mu\text{g/ml}$. Additionally, the apoptotic induction ability of frullanolide at various concentrations [0.5x, 1x and 2x half maximal inhibitory concentration (IC_{50})] was investigated by flow cytometry and western blot analysis. Frullanolide exhibited strong anti-breast cancer activity against MDA-MB-468 (IC_{50} , $8.04 \pm 2.69 \mu\text{g/ml}$) and weak cytotoxicity against the MCF-7 (IC_{50} , $10.74 \pm 0.86 \mu\text{g/ml}$) and MDA-MB-231 (IC_{50} , $12.36 \pm 0.31 \mu\text{g/ml}$) cell lines. The IC_{50} of frullanolide was high in the human normal epithelial breast cell line (MCF-12A) and mouse fibroblast cell line (L-929). Density plot diagrams revealed that frullanolide induced apoptosis in MCF-7, MDA-MB-468 and MDA-MB-231 cells. Notably, a plausible anticancer mechanism was elucidated via cellular apoptosis by p53-independence in the treated MCF-7 cell line and p53-dependence in the treated MDA-MB-468 and MDA-MB-231 cell lines. In conclusion, the present study

demonstrated that frullanolide may exert anticancer activity on breast cancer cell lines by inducing apoptosis. Frullanolide offers a possible novel approach to breast cancer therapy.

Introduction

Cancer is one of the leading causes of human mortality worldwide, with an estimated 14 million new cancer cases projected for 2030 (1). In women, breast cancer is quickly becoming the leading cause of mortality worldwide, and novel therapeutic avenues are constantly being explored (2). One such line of investigation involves evaluating natural products extracted from plants and endophytic fungi, such as vincristine and vinblastine, which have been demonstrated to exhibit anticancer activities, including the inhibition of breast cancer cell growth (3,4). Studies are continuously being conducted in the search for novel effective and nontoxic anticancer compounds from various medicinal plants.

The genus *Grangea* belongs to the Compositae (Asteraceae) family and comprises only six species, which are mostly native and distributed throughout Africa, South Asia and Southeast Asia (5,6). *G. maderaspatana* (L.) Poir. (Phayaa Mutti) is one of the most common medicinal plants used in traditional Thai medicine in various therapeutic approaches, including ingestion of the whole plant to stimulate digestion, reduce pain and inflammation, and regulate menses, while the leaf is used for reducing spasms (7). Although anesthetic, antioxidant, antibacterial and topoisomerase I and II (Top I and II) inhibitory activities have been reported previously for compounds derived from *G. maderaspatana* (8-12), to the best of our knowledge, no previous studies have assessed whether these compounds possess anticancer activity.

A previous study in our laboratory revealed the presence of sesquiterpene lactones (SLs) in *G. maderaspatana* (12). SLs are compounds which have several significant cancer-associated implications and are used in targeted therapy against cancer cells, their stem cells and specific signaling pathways (13). Various SLs, including thapsigargin, artemisinin and parthenolide, have been demonstrated to exhibit potent action against certain types of cancer (13). SL extracts, known

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as eudesmanolides, derived from frullanolide have been demonstrated to possess anticancer activity in oral, non-small cell lung and breast cancer cell lines (12). However, no scientific studies have been conducted concerning frullanolide and its anticancer activities or its cytotoxic mechanisms against breast cancer cells. Therefore, in the present study, the cytotoxic effects of frullanolide and its mode of action on breast cancer cell inhibition were explored.

Materials and methods

Cell cultures. The breast cancer cell lines MCF-7 (HTB-22TM), MDA-MB-468 (HTB-132TM) and MDA-MB-231 (HTB-26TM), and a normal epithelial breast cell line (MCF-12A; CRL-10782TM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained at the Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University (Hat Yai, Thailand). The mouse fibroblast L-929 (CCL-1TM; ATCC) cell line was provided by Professor Teerapol Srichana, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The culture conditions of the cell lines followed methods described previously (14). Briefly, MCF-7 cells were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the MDA-MB-468, MDA-MB-231 and L-929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc). The cultures were supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), with 100 U/ml of penicillin and streptomycin. The MCF-12A cell line was cultured in DMEM and Ham's F12 medium (GE Healthcare, Chicago, IL, USA), supplemented with 100 ng/ml cholera toxin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 500 ng/ml hydrocortisone (Sigma-Aldrich; Merck KGaA), 0.01 mg/ml bovine insulin (Sigma-Aldrich; Merck KGaA), 20 ng/ml human epidermal growth factor (Invitrogen; Thermo Fisher Scientific, Inc.) and 5% horse serum (Invitrogen; Thermo Fisher Scientific, Inc.). All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Plant material and isolation. Dried *G. maderaspatana* (L.) Poir plants were purchased from the Chaokromper Drug Store (Bangkok, Thailand). Their identity was confirmed by Dr Nijisiri Ruangrungsri, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Bangkok, Thailand). A voucher specimen (no. 5182) was deposited at the Museum of Natural Medicine, Chulalongkorn University. The dried whole plant materials were ground to coarse powder using a mortar and pestle. The powder was then stored at room temperature (RT) prior to extraction. Crude compounds were extracted with dichloromethane from a powder weight of 1,500 g. The extract was evaporated under vacuum at 55°C, fractionated by silica gel column chromatography (silica gel no. 9385) and then eluted with different gradients of hexane-ethyl acetate 10:0 to 0:10 solvent systems, resulting in 132.2 mg of a compound resembling white needles (Rf 0.85, silica gel CH₂Cl₂-Acetone 9:1). The structure of the compound was elucidated by ¹H, ¹³C nuclear magnetic resonance spectroscopy and mass spectrometry. Its physical and

spectral data were compared with previous reports (9,12). The compound was identified as the sesquiterpene lactone, frullanolide (Fig. 1), with the chemical formula C₁₅H₂₀O₂ (parent peak at m/z 232, colorless solid). The compound was stored at -20°C prior to testing with cancer cells.

MTT assay. All cell lines were seeded in 96-well plates at a density of 2x10⁴ cells/well in 100 µl culture medium/well. The cells were treated with different concentrations of the dimethyl sulfoxide (DMSO)-dissolved frullanolide compound (1.25, 2.5, 5.0, 10.0 and 20.0 µg/ml). After 72 h of incubation at 37°C, 100 µl MTT reagent (0.5 mg/ml) was added to each well and the cultures were incubated for an additional 30 min. The MTT reagent was removed and replaced by DMSO to ensure that solubilization was complete. Absorbance at 570 and 650 nm (reference wavelengths) was measured on a microplate reader. Half-maximal inhibitory concentration (IC₅₀) values were calculated from fitted response curves of the concentration and viability (%). Determination of cytotoxic activity followed the criterion; <5 µg/ml represented highly active; 5-10 µg/ml represented strongly active; and >10 represented weak cytotoxicity (15,16). Normal breast cells (MCF-12A) and L-929 fibroblasts were treated using the aforementioned procedure. Selectivity index (SI) values, indicating selectivity for tested cell lines, were calculated from the ratio of IC₅₀ values of the compounds obtained for normal vs. cancer cells. An SI score >3 represented good selectivity (17). This experiment was performed in triplicate.

Flow cytometry for cell cycle analysis and apoptosis detection. The MCF-7 and MDA-MB-468 cells were seeded at densities of 4x10⁵ cells/well in 12-well plates. The MDA-MB-231 cells were seeded at 3x10⁵ cells/well. The cells were treated for 24 h with three concentrations of frullanolide (0.5x, 1x and 2x IC₅₀). Following removal of the compound, the treated cells were fixed with 70% ethanol at 4°C for 4 h and washed three times with cold PBS. Harvesting and fluorochrome binding of the cells were carried out as described previously (2). For cell cycle analysis, the fixed cells were resuspended in 400 µl propidium iodide (PI; 50 µg/ml) solution/1x10⁶ cells and incubated at RT for 5-10 min in the dark. A total of 5,000 cells were analyzed for each condition with a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), and histograms of cell population ratios in each phase of the cell cycle were acquired. For apoptosis detection, the apoptotic cells in the treated conditions were stained with Annexin V-fluorescein isothiocyanate (FITC)/PI following the manufacturer's protocol (FITC Annexin V Apoptosis Detection kit I; BD PharmingenTM, BD Biosciences). Dot plot graphs of the apoptotic cell ratios were created. All data were analyzed using WinMDI v.2.9 software (J. Trotter, The Scripps Institute, La Jolla, CA, USA).

Western blot analysis. Untreated cells and cells treated with frullanolide at 0.5x IC₅₀, were harvested at 0, 12, 24 and 48 h. The cells were lysed in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples were quantitated by Bradford Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total proteins (50 µg) of each sample were run separately on a 12% SDS-PAGE

Table I. Cytotoxic activity (IC_{50}) of frullanolide purified from whole plant of *G. maderaspatana* exhibits differing dosage dependence in MCF-7, MDA-MB-468, MDA-MB-231, MCF-12A and L929 cell lines.

Cell line	IC_{50}^a , $\mu\text{g/ml}$ (μM)	Cytotoxic activity	SI ^b	SI activity
MCF-7	10.74 \pm 0.86 ^c (46.23)	Weak cytotoxicity	2.67	Less selectivity
MDA-MB-468	8.04 \pm 2.69 ^c (34.61)	Strongly active	3.56	High selectivity
MDA-MB-231	12.36 \pm 0.31 ^c (53.20)	Weak cytotoxicity	2.32	Less selectivity
MCF-12A	28.65 \pm 6.57 (123.32)	Weak cytotoxicity	None	None
L929	19.07 \pm 7.16 (82.08)	Weak cytotoxicity	None	None

IC_{50} values are presented as the mean \pm standard deviation. ^aActivity scores (IC_{50} $\mu\text{g/ml}$): <5 =highly active; 5-10=strongly active; >10 =weak cytotoxicity, considered cytotoxic against human breast cancer MCF-7, MDA-MB-468 and MDA-MB-231 cell lines (15,16); ^bSI >3 =good selectivity (17), SI activity against the epithelial normal breast MCF-12A cell line; ^cP <0.05 for independent IC_{50} values of cancer cells compared with normal cell line (MCF-12A). IC_{50} , half-maximal inhibitory concentration; SI, selective index.

gel and transferred onto a nitrocellulose membrane in glycine-methanol buffer at 4°C (100 V, 2 h). The membranes were blocked in 5% low-fat milk in TBS with 0.1% Tween-20 (TBS-T) at RT for 1 h. Subsequently, the blots were incubated at 4°C overnight with primary antibodies; B cell lymphoma 2-associated X protein (Bax; cat no. 5023; 1:1,000 dilution), p21 (cat no. 2947; 1:1,000 dilution), p53 (cat no. 9282; 1:1,000 dilution) and β -actin (cat no. 4967; 1:1,000 dilution), in 1% low-fat milk in TBS-T. All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The blots were then washed 3 times (5 min each) and incubated with enhanced chemiluminescence (ECL) anti-rabbit immunoglobulin G horseradish peroxidase (cat no. PKNA934; GE Healthcare Life Sciences, Little Chalfont, UK) diluted to 1:5,000 in 1% low-fat milk in TBS-T at RT for 1 h. The protein bands were detected using an ECL chemiluminescent detection kit (Thermo Fisher Scientific, Inc.). Due to the limited amount of frullanolide compound available, each treatment was performed in triplicate. The triplicate samples were pooled prior to protein lysate preparation and western blotting.

Statistical analysis. The MTT results are presented as the mean \pm standard deviation. To evaluate the difference between the cell lines, one-way analysis of variance (ANOVA) with Brown-Forsythe correction was performed, then IC_{50} values of the cancerous cell lines were compared with MCF-12A cells using one-sided Dunnett's post hoc tests. Statistical analysis was performed with SPSS 20.0 software (IBM Corp., Armonk, NY, USA). P <0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic activity of frullanolide. The IC_{50} values of frullanolide for the breast cancer cell lines MCF-7, MDA-MB-468 and MDA-MB-231 were 10.74 \pm 0.86, 8.04 \pm 2.69 and 12.36 \pm 0.31 $\mu\text{g/ml}$, respectively. The IC_{50} value for normal breast cells (MCF-12A) was 28.65 \pm 6.57 and 19.07 \pm 7.16 $\mu\text{g/ml}$ for the fibroblast L-929 cell line. One-way ANOVA indicated significantly different values among the groups. It was tested whether the compound had lower IC_{50} values in the cancerous cell lines than when applied to MCF-12A cells. In cancerous cell

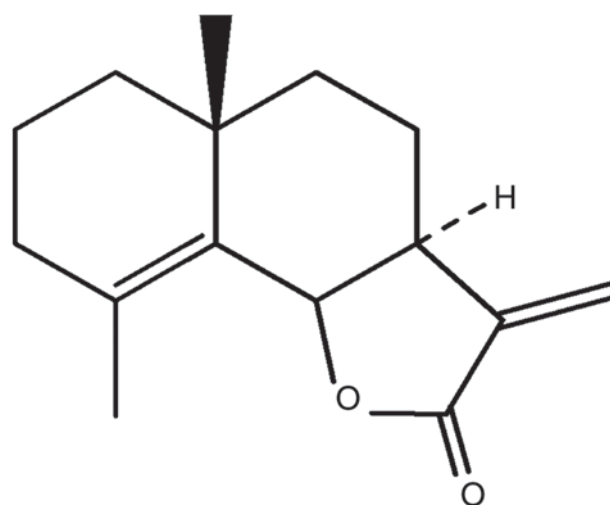


Figure 1. Chemical structure ($C_{15}H_{20}O_2$) of sesquiterpene lactone (frullanolide) isolated from whole plant material of *G. maderaspatana* (L.) Poir.

lines, frullanolide had significantly lower IC_{50} values compared with MCF-12A, whereas the L-929 fibroblast cell line did not exhibit a significant difference. The results indicated a strong anti-proliferative effect of frullanolide on the breast cancer cell lines, but less of an effect on normal breast and fibroblast cell lines (Table I). The SI, which indicates the safety level of a compound toward normal breast cells, indicated that frullanolide has high cytotoxic activity against MDA-MB-468 (SI=3.56) but was less harmful to normal breast cells.

Cell cycle arrest of frullanolide. Cell cycle arrest was monitored using flow cytometry (Figs. 2 and 3). Following staining of the nuclei with PI, the FACS analysis revealed that frullanolide induced an increased proportion of SubG₁ cell debris for the MCF-7, MDA-MB-468 and MDA-MB-231 cell lines in a dose-dependent manner. The percentage of SubG₁ cells in the MCF-7 cell line increased from 79.77 at 0.5 $\times IC_{50}$ to 90.10% at 2 $\times IC_{50}$, compared with 51.05% in untreated cells (Figs. 2A and 3A). There was also an increase in SubG₁ cells in MDA-MB-468, from 50.97 at 0.5 $\times IC_{50}$ to 58.60% at 2 $\times IC_{50}$ dosage (Figs. 2B and 3B). Additionally, the SubG₁ proportion

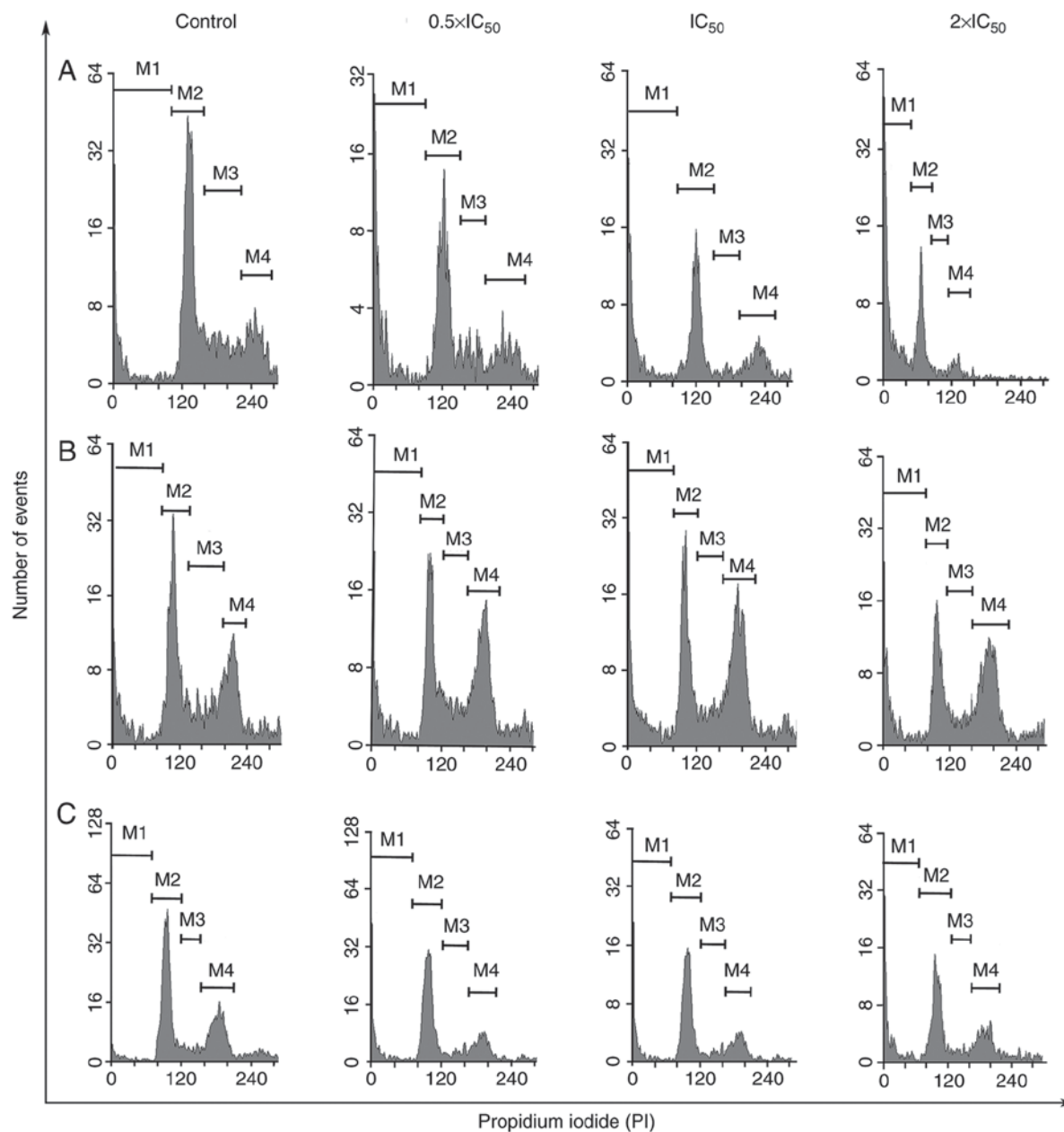


Figure 2. Cell cycle distribution of breast cancer cell lines following frullanolide treatments (0.5x, 1x and 2xIC₅₀) using flow cytometry analysis. Histograms of PI-labelled DNA content of each cell cycle phase in untreated and treated (A) MCF-7, (B) MDA-MB-468 and (C) MDA-MB-231 cells, respectively. M1, M2, M3, M4 in each histogram designate the number of cells in the respective SubG₁, G₁, S and G₂/M phases. IC₅₀, half-maximal inhibitory concentration; PI, propidium iodide.

of the MDA-MB-231 cell line increased from 59.31 at 0.5xIC₅₀ to 76.62% at 2xIC₅₀ dose levels, compared with 37.47% in untreated cells (Figs. 2C and 3C). The cell proportions in the G₁, S and G₂/M phases in the histograms of the MCF-7 treated cells tended to decrease in a dose-dependent manner, from 11.87 to 6.91 (G₁), 3.22 to 1.14 (S) and 5.14 to 1.85% (G₂/M) (Figs. 2A and 3A). The distribution of MDA-MB-468 cells among the phases also changed in a dose-dependent manner (Fig. 2B). For MDA-MB-468, the number of treated cells in the G₁ and S phases gradually decreased until the highest dosage (2xIC₅₀). Conversely, cells appeared to accumulate in the G₂/M phase in treated cells (20.43, 24.13 and 20.37% at 0.5x, 1x and 2x IC₅₀, respectively), compared with untreated cells (14.81%; Fig. 3B). Notably, the G₁, S and G₂/M phases of the MDA-MB-231

cell cycles slightly decreased in a dose-dependent manner from 0.5x to 2xIC₅₀ dose levels (G₁, 25.53 to 13.19; S, 5.06 to 2.52; and G₂/M, 10.10 to 7.68%) as shown in Figs. 2C and 3C. These results indicated that frullanolide was associated with cell cycle arrest at G₂/M in MDA-MB-468 but not in the MCF-7 and MDA-MB-231 cell lines.

Apoptosis induction of frullanolide. To assess the apoptotic action of frullanolide, Annexin V-FITC/PI was used to stain treated cells. A density plot was created based on the data obtained from the FACS analysis. The plot was divided into four quadrants (Fig. 4A-C), with the lower left quadrant presenting viable cells (Annexin V-negative, PI-negative), the lower right quadrant presenting cells that underwent early

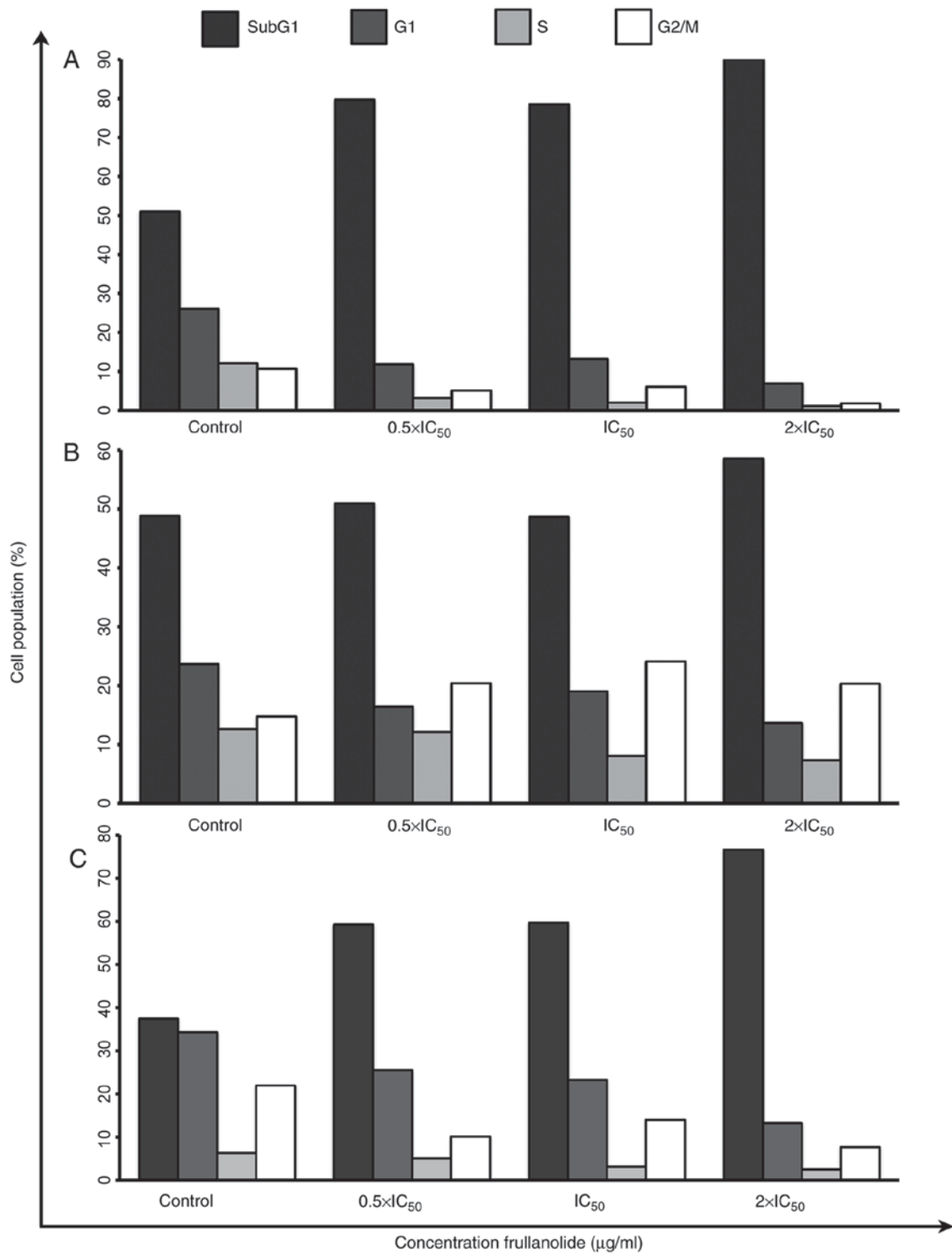


Figure 3. Alterations in the number of cells (%) in each stage (SubG₁, G₁, S and G₂/M) in untreated and treated breast cancer cell lines (0.5x, 1x and 2xIC₅₀ of frullanolide). The quantitative data obtained from the Fluorescence Activated Cell Sorter assay represent different cells in each cell division in treated (A) MCF-7, (B) MDA-MB-468 and (C) MDA-MB-231 cells. IC₅₀, half-maximal inhibitory concentration.

apoptosis (Annexin V-positive, PI-negative), the upper right quadrant presenting cells which underwent late apoptosis (Annexin V-positive, PI-positive), and the upper left presenting necrotic cells (Annexin V-negative, PI-positive). After 24 h of treatment of MCF-7 cells, the lowest viable counts along with the highest apoptotic counts (early and late apoptosis) were identified at the 0.5xIC₅₀ dose level (14.97 and 84.38%, respectively; Fig. 4A and D). These results indicated that

frullanolide may be effective in MCF-7 cells at different dose levels, particularly at a low dose level (0.5xIC₅₀). Conversely, frullanolide treatment of MDA-MB-468 cells resulted in the lowest number of viable cells (11.96%) and the highest number of apoptotic cells (86.38%) at the highest dosage (2xIC₅₀; Fig. 4B and E). Although no significant differences were observed for the MDA-MB-231 cells regarding the number of viable and apoptotic cells among all treatment conditions,

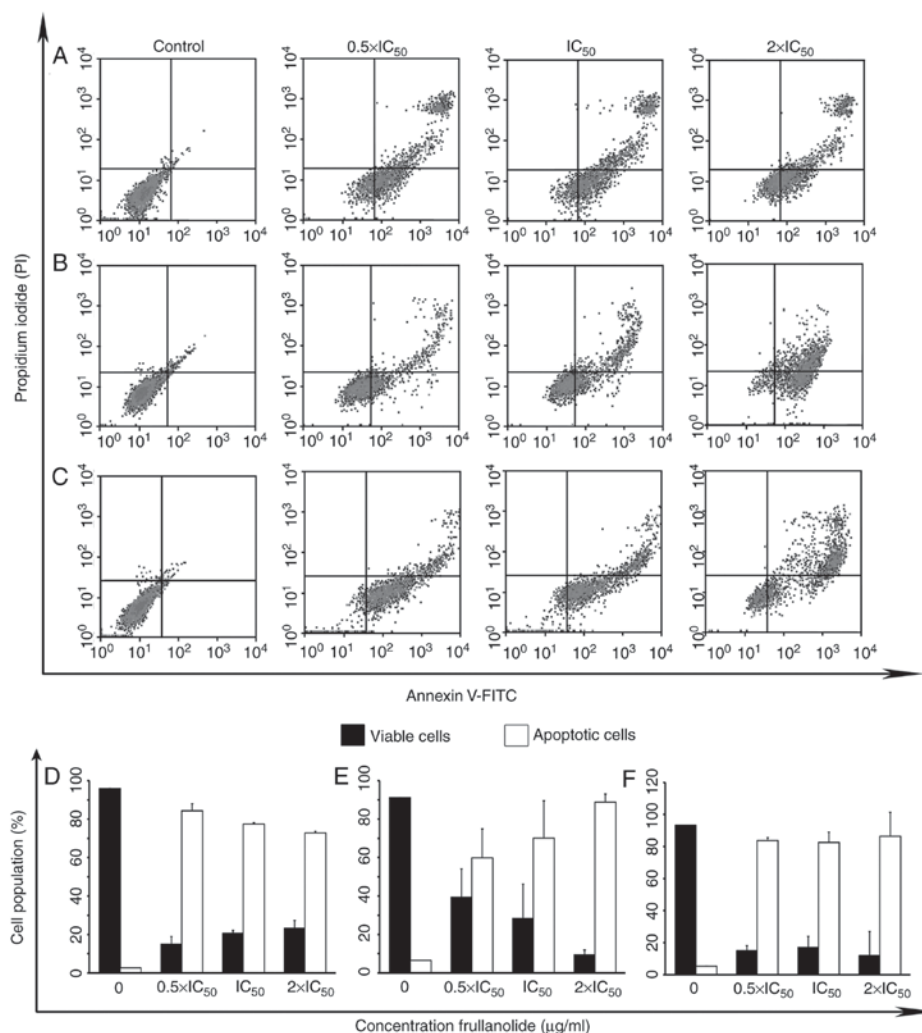


Figure 4. Density plots of frullanolide-induced apoptosis. (A) MCF-7, (B) MDA-MB-468 and (C) MDA-MB-231 cells treated with frullanolide at 0.5x, 1x and 2xIC₅₀. The density plots show four quadrants; the lower left, lower right, upper right and upper left quadrants represent viable, early apoptotic, late apoptotic and necrotic cells, respectively. The percentages of cell populations indicate viable cells (black column) and total apoptotic cells (white column) for the (D) MCF-7, (E) MDA-MB-468 and (F) MDA-MB-231 cell lines. Error bars represent the data obtained from treated breast cancer cells in two independent experiments. FITC, fluorescein isothiocyanate; IC₅₀, half-maximal inhibitory concentration; PI, propidium iodide.

treatment exhibited effective cytotoxic activity compared with the control (Fig. 4C and F). Therefore, the data indicated that frullanolide may induce apoptosis in breast cancer cells at all tested concentrations.

Protein expression by western blot analysis. To elucidate the possible mechanisms of apoptotic induction by frullanolide, the expression levels of three proteins (Bax, p21 and p53) were investigated by western blot analysis at 0.5xIC₅₀, for 12, 24 and 48 h in the three breast cancer cell lines. The overall effect of the frullanolide treatment in these breast cancer cell lines on detected protein expression is presented in Fig. 5. For all cell types treated with 0.5xIC₅₀ frullanolide, Bax protein expression decreased between 12 and 24 h. Its expression was prone to accumulation in treated MCF-7 and MDA-MB-231 cells at 48 h (Fig. 5A, C, J and L). However, expression levels of Bax protein increased at 12 h in treated MDA-MB-468 cells and its expression gradually reduced at 24–48 h (Fig. 5K). Elevated p21 expression was noted at 12 h, and gradually decreased between 24 and 48 h in all three breast cancer cell lines. The p21 expression was upregulated when

compared with the controls (Fig. 5G-I). Notably, p53 protein was expressed differentially depending on the cell type. p53 expression was undetectable in MCF-7 cells (Fig. 5A and D), while distinct adverse alterations were identified in the treated MDA-MB-468 and MDA-MB-231 cells (Fig. 5E and F). The p53 protein levels in frullanolide-treated MDA-MB-468 cells increased at 12–24 h, and immediately declined by ~0.80-fold until the end of exposure (24–48 h) when compared with the control group (Fig. 5E). Therefore, it appeared conclusive that the frullanolide mechanism was involved in apoptotic pathways.

Discussion

SLs, derived from a natural product and a subfamily of terpenoids, have been reported to exhibit numerous potential medicinal properties, including anti-inflammatory, antimicrobial and anticancer actions, in *in vitro* and *in vivo* studies, as well as clinical trials (13,18–20). More than 5,000 SLs have been identified from *Asteraceae spp.* (18), and ~1,500 publications have reported on their anti-inflammatory and anticancer

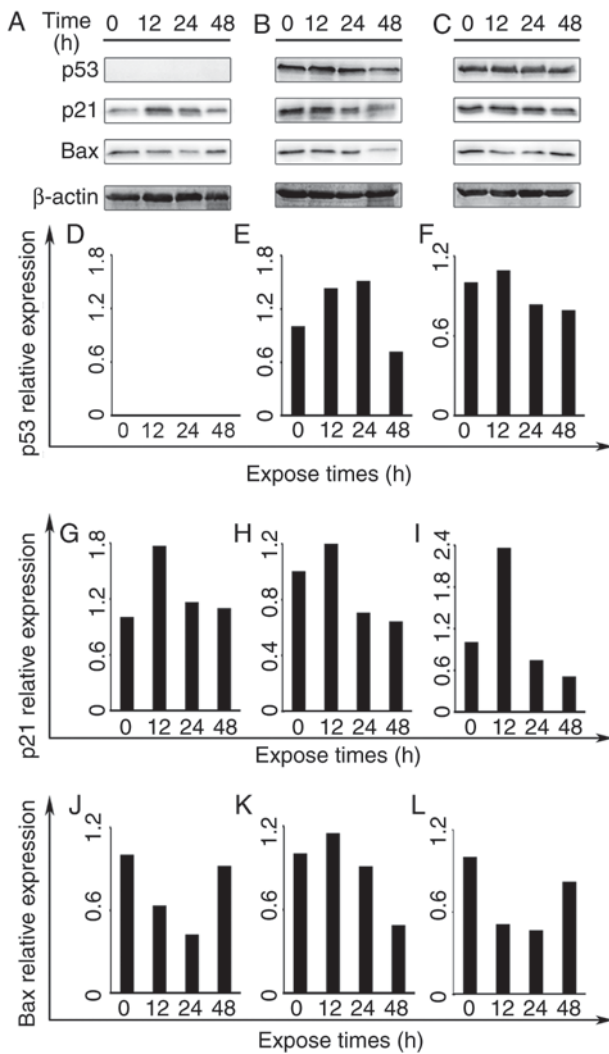


Figure 5. The expression of apoptosis-associated proteins in treated breast cancer cell lines. (A) MCF-7, (B) MDA-MB-468 and (C) MDA-MB-231 cells treated with 0.5x IC₅₀ frullanolide for 12, 24 and 48 h. The bar charts represent the relative expression of (D-F) p53, (G-I) p21 and (J-L) Bax proteins at the indicated times in all frullanolide treated-breast cancer cell lines. Bax, B cell lymphoma 2 associated X protein; IC₅₀, half-maximal inhibitory concentration.

properties (13). *In vitro* cytotoxicity screening for antitumor agents has been widely employed in various types of cancer cell lines in the search for novel anticancer drugs. A number of potentially active compounds have been identified through a large-scale screening program based on the US National Cancer Institute criteria (21). Purified and crude compounds of one of these anticancer agents were reported to possess strong antitumor activity, with IC₅₀ values of ≤ 4 and ≤ 20 $\mu\text{g/ml}$, respectively (21). Several studies have identified specific SLs and derivatives which exhibit broad-spectrum antitumor activity towards several cancer types, including non-small cell lung cancer, colorectal cancer, leukemia, laryngeal cancer, gynecological cancer and breast cancer (13,22-27). The different anticancer abilities of SLs are associated with their carbocyclic skeleton classification (13,28). Eudesmanolides (6/6-bicyclic compounds) a subgroup of SLs, have been reported in several studies to exhibit anticancer action (29-34). For example, the eudesmane skeleton (santamarine) has

been revealed to exhibit potential anticancer properties toward the L1210 murine (IC₅₀=0.41 $\mu\text{g/ml}$), CCRF-CEM human leukemia (IC₅₀=0.59 $\mu\text{g/ml}$), KB human nasopharyngeal (IC₅₀=0.16 $\mu\text{g/ml}$), LS174T human colon carcinoma (IC₅₀=0.92 $\mu\text{g/ml}$) and MCF-7 breast adenocarcinoma (IC₅₀=0.53 $\mu\text{g/ml}$) cell lines (29). Li *et al* (30), studied the efficacy of eudesmane-based santamarine against a number of gynecological cancer cell lines, and revealed that HeLa ovarian cancer and SHIN3 cervical cancer cell line viabilities are decreased by 50% following respective treatments of 2.60 and 3.08 $\mu\text{g/ml}$ santamarine for 48 h, while the HOC-21 and HAC-2 ovarian cancer, and HEC-1 endometrial cancer cell lines had some tolerance (IC₅₀>10 $\mu\text{g/ml}$) (31). Similarly, the cytotoxic activity of a eudesmanolide compound [15-hydroxy-eudesm-4,11(13)-diene-12-oic acid] is potent toward a panel of human cancer cell lines (PC prostate cancer, HT29 colon cancer, MCF-7 breast cancer and A549 lung cancer) with IC₅₀ of 5.8±0.2, 5.8±0.2, 6.8±0.4 and 69.6±7.1 μM , respectively (31). Five compounds isolated from the flowers of *Tanacetum vulgare* exhibit degrees of anticancer activity between 15.3 and 60.0 μM against A549 lung cancer cells (32). Notably, a novel 12,8-eudesmanolide extract from *Eutypella spp.* exhibits cytotoxicity against lymphoma, hepatocarcinoma and myeloid leukemia cell lines (33-34). These and other studies indicate that eudesmanolide-based SLs are potentially strong anticancer compounds for the treatment of various types of cancer, including breast cancer.

In the present study, frullanolide (a eudesmanolide) was assessed by MTT assay, and exhibited potent anti-breast cancer activity in breast cancer cell lines, including MCF-7, MDA-MB-468 and MDA-MB-231. The IC₅₀ values were measured after 72 h. In comparison, the cytotoxicity of frullanolide was strongest for MDA-MB-468, a triple negative breast cancer (TNBC) cell line which lacks estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (35). This TNBC cell line is an important model for breast cancer responsiveness due to the limited choices of chemotherapeutic treatment for this disease (35-37). Notably, in the present study, frullanolide exhibited potent activity against MDA-MB-468 cells, which possess a unique targeted receptor, the epidermal growth factor receptor (EGFR), on the cell membrane (35). This receptor may be involved in the specific interactions with frullanolide, leading to its toxicity in these cells. Using *in silico* screening, Sawatdichaikul *et al* (38) reported a list of plant compounds that bind to EGFR. Two promising compounds from the medicinal plants list are exiguafavanone A and exiguafavanone B, which are compounds purified from *Artemisia indica* Willd and *Sophora exigua*, respectively. These belong to the Asteraceae family, the same family as *G. maderaspatana* (L.) Poir. The structure of these two compounds has approximately the same carbon skeleton orientation as frullanolide (38). However, further experiments are required to confirm any interactions between frullanolide and EGFR. Additionally, Maldonado *et al* (27) reported on two novel eudesmanolide structures (C₁₇H₂₅O₅ and C₁₇H₂₂O₄) extracted from the small genus *Kaunia* (Asteraceae family) which exhibited strong anti-breast cancer activity toward five breast cancer cell lines; HCC1937 (TNBC), JIMT-1, L56Br-C1, MCF-7 and SK-BR-3. The IC₅₀ values of eudesmanolide C₁₇H₂₅O₅

and C₁₇H₂₂O₄ ranged between 9.3 and 27.0 μ M (L56Br-C1 >SK-BR-3 >JIMT-1 >HCC1937 >MCF-7) and 3.2-11.0 μ M (HCC1937 >SK-BR-3 >JIMT-1 >L56Br-C1 >MCF-7) (27). A previous computational structure-based screening method to identify natural compounds that specifically target the mouse double minute 2 homolog protein revealed that, out of the 35 top candidates, 8 eudesmanolide SLs (IJ-1, IJ-3, IJ-5, IJ-6, IJ-9, IJ-11, IH-45 and IH-49) exerted more potent anti-TNBC activity (MDA-MB-231) than anti-non-TNBC (MCF-7) activity at 72 h (39). The IC₅₀ values ranged between 10 and 50 μ M (39). In the present study, the anti-breast cancer activity of frullanolide in the MDA-MB-468, MCF-7 and MDA-MB-231 cell lines exhibited similar IC₅₀ levels of 34.61, 46.23 and 53.20 μ M, respectively. In comparison, the anti-breast cancer activity of frullanolide in certain cell lines (specifically TNBC) exhibited moderately strong activity, but exhibited high selectivity for breast cancer cells (less harmful to normal breast cells).

To evaluate the mechanism of apoptosis following frullanolide treatment, flow cytometry analysis was selected for measurement of quantitative DNA content (PI staining) and apoptotic cells (double-staining fluorescent PI and annexin V-FITC). Apoptosis-associated protein (p53, p21 and Bax) expression levels were analyzed by western blotting. Notably, apoptotic induction by the appropriate frullanolide treatment occurred in all three breast cancer cell lines, but G₂/M arrest only occurred in frullanolide-treated MDA-MB-468 cells. Niculescu *et al.* (40), reported that the level of p21 accumulation serves a role in negatively regulating the G₂/M transition. The same study revealed that the expression of p21 is associated with endoreduplication in pRb-negative cells by inhibiting cyclin-dependent kinases leading to G₂ arrest (40).

The findings of the present study supported the observation of cell cycle arrest in MDA-MB-468, in that an increase of p21 expression after 12 h of treatment was observed. The expression of p21 also induces the apoptotic pathway in p53-dependent and -independent pathways (41). Overall, after 12 h of treatment, apoptosis was induced by increasing the expression of p21 and Bax at various time points in the MDA-MB-468, MDA-MB-231 and MCF-7 cell lines.

In the present study, p53 expression in treated MCF-7 cells was not detectable due to its short half-life, caused by proteasomal degradation (42,43). This effect may be regulated by p53-independent p21 activation molecules, including transforming growth factor β , tumor necrosis factor α , histone deacetylase inhibitors, interferon γ and interleukin 6 (41).

In conclusion, the results of the present study suggested that the apoptotic pathway was involved in frullanolide-induced cell death via p21 induction and p53-independent pathways in the MCF-7 cell line and p53-dependent pathways in the MDA-MB-468 and MDA-MB-231 breast cancer cell lines. Further experiments are required to clarify the molecular mechanism involved in the anti-breast cancer activity of frullanolide.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

SC performed the experiments, acquired and analyzed the data, and was a major contributor in the manuscript writing. PG was responsible for the cytotoxic activity assay and interpreted the data. TS provided the protocol and guidance for FACS analysis. SS extracted and purified the frullanolide compound. RB performed the western blotting. KK designed all the experiments, analyzed the data and was a major contributor in editing the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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