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Original Paper

Comprehensive Analysis of the Chemical Composition and *In Vitro* Cytotoxic Mechanisms of *Pallines Spinosa* Flower and Leaf Essential Oils Against Breast Cancer Cells

Ayman M. Saleh[a] Mahmoud A. Al-Qudah[b] Amre Nasr[c] Sayed A. Rizvi[d] Anwar Borai[a,e] Mustafa Daghistani[a,e]

[a]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), and King Abdullah International Medical Research Center (KAIMRC), King Abdulaziz Medical City, Ministry of National Guard Health Affairs, Jeddah, Kingdom of Saudi Arabia; [b]Department of Chemistry, Faculty of Science, Yarmouk University, Irbid, Jordan; [c]Department of Basic Medical Sciences, College of Medicine, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), National Guard Health Affairs, Riyadh, Kingdom of Saudi Arabia; [d]Department of Pharmaceutical Sciences, College of Pharmacy, Nova Southeastern University (NSU), Fort Lauderdale, Florida, USA; [e]Department of Pathology and Laboratory Medicine, King Abdulaziz Medical City, Ministry of National Guard Health Affairs, Jeddah, Kingdom of Saudi Arabia

Key Words
Pallines spinosa • Essential oils • Anticancer natural compounds • Apoptosis • Cell cycle • G0/G1 phase • Cyclin-D • CDK4 • p21 • Breast cancer cell lines

Abstract
Background/Aims: In our quest for new natural anticancer agents, we studied the cytotoxicity of the essential oils extracted from flowers and leaves of *Pallines spinosa*. ***Methods:*** The essential oils were extracted by hydrodistillation and solid phase microextraction (SPME) from flowers and leaves of the plant and their composition was determined by GC/GC-MS. The cytotoxicity of the oils was evaluated against MCF-7 and MDA-MB-231 breast adenocarcinomas, and the non-cancerous MCF-10-2A cells, using a flow cytometry-based assay. Apoptosis was evaluated by flow cytometry, nuclear staining, caspases activation, and Western blotting techniques, and cell cycle by measuring DNA contents. ***Results:*** The hydrodistilled flower oil contained mainly sesquiterpenes (96.39%), while the leaf sample was dominated by oxygenated-sesquiterpenes (51.60%) and sesquiterpene-hydrocarbons (34.06%). In contrast, the SPME oil contained mainly monoterpene-hydrocarbons (44.09%) and sesquiterpene-hydrocarbons (34.15%) in the flower and leaf samples, respectively. The cytotoxicity of the flower oil against MCF-7 (IC_{50} 0.25 ± 0.03 µg/mL) and MDA-MB-231 (IC_{50} 0.21 ± 0.03 µg/mL) was much stronger than the leaf oil (IC_{50} 2.4 ± 0.5 µg/mL and 1.5 ± 0.1 µg/mL, respectively). The toxicity of the flower oil was ~5 to 8-times less in normal MCF-10-2A (IC_{50} 1.3 ± 0.2 µg/mL) and blood mononuclear cells (2.80 ± 0.45 µg/mL) as compared to breast and hematological cancer cells, respectively.

Ayman M Saleh
and Mahmoud A Al-Qudah

King Saud Bin Abdulaziz University for Health Sciences-Jeddah Saudi Arabia)
and Yarmouk University-Irbid (Jordan)
E-Mail salehay@ksau-hs.edu.sa/mahmoud.qudah@yu.edu.jo

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Both oils induced a caspase-dependent and -independent apoptosis in MCF-7 and MDA-MB-231 cells, and altered the levels of Bcl-2 and Bax proteins. In addition, the oils arrested cell cycle in both cancer cell lines at G0/G1 phase by modulating the expression of cyclin D1, CDK4 and p21 proteins. ***Conclusion:*** The cytotoxicity of *P. spinosa* oils were mediated by apoptosis and cell cycle arrest, suggesting the potential use of their bioactive compounds as natural anticancer compounds.

Introduction

Breast cancer is one of the most reported tumors, and it is the second leading cause of cancer mortality in women worldwide, with more than 255,000 (highest among all types) new cases expected in the United States in 2017 [1, 2]. The ductal carcinoma is the most common type (90%) of non-invasive breast cancer, while lobular carcinoma and inflammatory breast cancer are also reported [3-5]. There are many factors identified in its pathogenesis, including, genetic predisposition, alcohol consumption, diet and obesity, physical inactivity, and endocrine factors [3, 6]. The most common recommendation of breast cancer management is the surgical removal of the lump (lumpectomy), or the entire breast (mastectomy) depending on the stage. Other treatment modalities include radiation therapy and chemotherapy, they can both be used prior to and after the surgery [6].

Treatment with cytotoxic chemotherapy in both early and advanced stages of breast cancer has made significant progress in the last decade with substantial key studies showing better survival benefits of newer therapies [7]. In spite of improvements with recent synthetic and adjuvant therapies for early stage breast cancer, resistance to therapy remains a major challenge in the management of advanced stages of the disease [7]. Therefore, better understanding of the molecular signaling basis of breast cancer should provide further potential targets for novel therapies.

Alteration of apoptosis, the highly controlled process of programmed cell death, is the main cause of cancer and it is associated with poor chemotherapy outcomes [3]. Herceptin Receptor 2 (HER2) is a trans-membrane receptor tyrosine kinase that activates multiple growth-promoting signaling pathways with a frequently occurrence in breast cancer that causes tumor resistance to treatment with cytotoxic regimen [8]. Hyperactivation or overexpression of this receptor is the primary cause of suppressing apoptosis which leads to enhancing cell survival giving rise to uncontrolled proliferation and tumor growth [8]. Both the extrinsic and intrinsic pathways of apoptosis have been shown to be disrupted in HER2 overexpressing breast cancer cells. HER2 overexpression is associated with increased expression of survivin and decreased response to signaling by cell surface death receptors leading to inhibition of caspases involved in activating the extrinsic pathway of apoptosis [9, 10]. HER2 also increases the expression of anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL, and Mcl-1), and therefore inhibits the release of cytochrome c from the mitochondria to activate the Apaf-1/caspase-9 apoptosome [11]. Moreover, HER2 is able to translocate and sequester cytochrome c in the mitochondria to prevent its release [12]. HER2 also suppresses p53-mediated apoptosis through upregulating the E3 ubiquitin-protein ligase MDM2 [13].

In addition to evading apoptosis, dysregulation of the cell cycle is a highly common feature of proliferating and metastatic breast tumor cells [14]. Cyclin-dependent kinases (CDKs) are critical regulators of cell cycle progression and their activity is regulated through association with different regulatory cyclin subunits and endogenous cyclin dependent kinase inhibitors (CKIs), as well as by positive and negative phosphorylation events [14]. In malignant mammary cells, altered expression of CDKs and their modulators, including overexpression of cyclins and decreased expression of CKIs result in deregulated CDK activity leading to loss of cell checkpoint integrity and ultimately uncontrolled cell proliferation [14].

As a way of defensive mechanism, some plants produce toxic secondary metabolites. Some of these compounds pose wide array of pharmacological activities and even found

to be more toxic for cancer cells compared to the normal cells [15]. These compounds often become a source of new and better drugs via rational design in medicinal chemistry [15]. Essential oils are composed of low molecular weight aromatic compounds such as terpenoid hydrocarbons, oxygenated terpenes, and sesquiterpenes [16]. There are about 3000 essential oils reported and 10% (~300) of those are used in various pharmaceutical, cosmetics, agrochemical and food industries. Recently, extensive efforts have been used in evaluating the cytotoxic capacity of essential oils, and particular focusing on those modulating apoptosis and cell cycle progression for their potential applications in cancer prevention and treatment [15, 16].

The classical techniques for the determination of plant essential oils composition utilize two steps: extraction (solvent-based, steam distillation, hydrodistillation and solid phase microextraction, SPME) and both qualitative and quantitative analysis (gas chromatography (GC) or GC-mass spectrometry (GC-MS) [17]. The identity and possibly the quantity of the compounds in essential oils depend on the extraction method used. Solvent extracted oil may contain non-volatile compounds that affect the analysis step by GC and GC-MS. Steam distillation process is performed at 100°C causing loss of several volatile components. Although hydrodistillation technique is more preferred over steam distillation, it has the disadvantage of potential thermal degradation of some compounds due to the long heating period during extraction. On the other hand, the headspace SPME (will be referred here as SPME) is the most advanced technique that is able to extract and concentrate analytes of different volatility, including trace essential oil substances from a very little amount of plant material [17]. However, SPME is limited to compositional analysis rather than providing essential oil for other purposes.

Pallenis spinosa (L.) de Cassini (family; Asteraceae), commonly known as the Spiny Starwort, is an annual herbaceous plant that grows in vast areas of Jordan and the Middle East region [18]. This plant has been known and used for the treatment of eczema, rheumatism, muscular contraction, gastralgia, diabetes, headaches, skin injuries, and infections [19, 20]. Secondary metabolites identified in this plant include sesquiterpenes and oxygenated sesquiterpenoids [21]. The hydrodistilled volatile oil from *P. spinosa* flower-heads collected in Italy contains high percentages of oxygenated sesquiterpenoids, such as germacra-1(10),5-dien-3,4-diol, α-cadinol, 3-acetoxygermacra-1(10),5-dien-4-ol, T-cadinol and δ-cadinene [21]. However, up to date, the biological activities of the plant have not been documented.

In the present study we aimed to determine the detailed composition of *P. spinosa* flower and leaf essential oils extracted by hydrodistillation and SPME, and evaluate their anticancer potential against the human hormone-sensitive MCF-7 and -insensitive MDA-MB-231 breast cancer cells. This report also aimed to confirm that the cytotoxic potential of the oils was mediated by apoptosis and cell cycle disruption in both cancer cell lines.

Material and Methods

Reagents

GC grade *n*-hexane, C_8-C_{20} *n*-alkanes, dimethyl sulfoxide (DMSO), doxorubicin, RIPA cell lysis buffer, Tris-hydrochloride, bovine serum albumin (fraction V), protease inhibitors (PMSF, pepstatin A, leupeptin, and aprotinin) and Hoechst 33342 stain were acquired from Sigma Aldrich (St Louis, Missouri, USA). Anhydrous sodium sulfate was purchased from UCB (Bruxelles, Belgium). Cell culture media (DMEM, DMEM/F12, IMDM, RPMI 1640 and Leibovitz's L-15), penicillin-streptomycin, trypsin, phosphate buffered saline (PBS), heat inactivated fetal bovine serum (FBS), horse serum, insulin, epithelial growth factor (EGF), cholera toxin, hydrocortisone, sodium pyruvate, Glutamax® and non-essential amino acids were purchased from Invitrogen (Invitrogen, Carlsbad, California, USA). Cell culture grade disposable and materials are from Corning Life Sciences (Tewksbury, Massachusetts, USA). The colorimetric tetrapeptide substrate for caspas-3/-7 (Ac-DEVD-*p*NA) and the general caspase inhibitor (z-VAD-FMK) were purchased from BioVision, Inc. (Milpitas, California, USA). The primary antibodies against [cleaved form of caspase-3 (p19) and poly-ADP-ribose polymerase (PARP), from apoptosis antibody sampler kit # 9915], Bcl-2 family members; [(Bcl-2 and Bcl-X_L), from the pro-survival Bcl-2-family antibody sampler kit # 9941] and

[(Bax and Bak), from the pro-apoptosis Bcl-2-Family antibody sampler kit # 9942], cyclins [(cyclin D1, cyclin D2, cyclin D3 and cyclin E), from cyclin antibody sampler kit # 9869], cyclin-dependent kinases (CDK2, CDK4, CDK6) and cyclin dependent kinase inhibitors (p21 and p27) [from cell cycle regulation antibody sampler kit # 9932] were all obtained from Cell Signaling Technology (Beverly, Massachusetts, USA), while the one for β-actin was from Sigma Aldrich (# A5316). The secondary anti-mouse (# 7076) and anti-rabbit (# 7074) IgG antibodies conjugated to Horseradish peroxidase (HRP) were from Cell Signaling Technology.

Plant material and extraction of essential oils

Fresh flowers and leaves of *Pallenis spinosa* were collected from "Kufor Asad" Town, located 15 Km to the west of Irbid City in northern area of Jordan, in April, 2015. The plant was classified by a botanist, and a voucher specimen (No YU/68/CP/1001) was deposited in the herbarium of the Department of Biological Sciences, Yarmouk University, Irbid, Jordan as a reference. 300 g of the plant flowers and leaves were washed with double distilled water, then directly chilled in liquid nitrogen and powdered using a mortar and pestle. Subsequently, each powdered plant material was hydrodistilled in 300 mL distilled water using a Clevenger apparatus [22]. The hydrodistillation process was continued for 3 h after appearance of first drop of distillate. The collected oil was dried over anhydrous sodium sulfate to remove any residual water, filtered and then immediately stored in GC-grade *n*-hexane at 4 ºC until used for GC and GC-MS analysis. The hydrodistilled essential oil yield was 0.12% from the flowers (F-PSEO) and 0.08% w/w from the leaves (L-PSEO) of the plant. The density of F-PSEO and L-PSEO was 0.9352 g/mL and 0.9275 g/mL, respectively. For all the biological assays down below, stock solutions of 1000X of different concentrations of F-PSEO and L-PSEO were prepared in DMSO and stored at -20°C until used.

SPME of volatile oils was performed manually using a partially cross-linked polydimethylsilocane/divenylbenzene (PDMS/DVB) fiber assembly (1.0 cm long and a 65 µm thick, Supelco Inc., Bellefonte, Pennsylvania, USA). The fiber was conditioned as per the manufacturer's instructions. The fiber was immersed in a special tightly capped 4.0 mL amber glass vial containing 0.1 mg of freshly minced flower or leaf of *P. spinosa* to trap the volatile compounds. Trapping and concentration of the volatile constituents by the fiber was allowed to take place for 2 min inside the vial at room temperature. Subsequently, the analytes were desorbed at 240°C for 2 min and analyzed by GC–MS.

GC and GC-MS Analysis of oil composition

Aliquots, 1 µL of each oil sample, were diluted to 10 µL of GC-grade *n*-hexane, and subjected to a coupled GC-MS analysis using Varian Chrompack CP-3800 GC/MS/MS-200 (Saturn, Netherlands) equipped with DP-5 (5 % diphenyl, 95 % dimethyl polysiloxane) GC capillary column (30 m × 0.25 mm i.d., 0.25 µm film thicknesses), with helium as an auxiliary carrier gas at a flow rate of 0.9 mL/min. The actual temperature in MS source reached 180°C and the ionization voltage was 70 eV. The column temperature was kept at 60°C for 1 min, and then programmed to rise to 246°C at a constant rate of 3°C/min, then maintained at 246°C for 3 minutes. A hydrocarbon mixture of *n*-alkanes (C8-C20) was analyzed separately by GC-MS under similar chromatographic conditions using the DP-5 column. Identification of the oil components was achieved by comparing their relative retention indices (RI) with known mass spectral data [23], the data base National Institute of Standards and Technology (NIST, Gaithhersburg, MD) [24] and our own libraries. A mixture of *n*-alkanes (C8-C20) was used as reference for the calculation of relative retention indices (RI) in temperature-programmed run using column of identical polarity [22]. Quantitative analysis (% of area) was performed using Hewlett-Packard HP-8590 gas chromatograph (Hewlett-Packard Co., Palo Alto, California, USA) equipped with a split-splitless injector (split ratio 1:50) and a FID detector. An optima-5 (5 % diphenyl, 95 % dimethyl polysiloxan) fused silica capillary column (30.0 m × 0.25 mm, 0.25 µm film thickness; Sorbent Technologies Inc., Norcross, Georgia, USA) was used. The temperature of the oven was gradually increased at a steady rate of 10°C/min between 60°C and 250°C then held at 250°C for 5 min. The temperature of the injector port and detector was maintained at 250°C and 300°C, respectively. The relative peak areas of the oil components were measured and used to extrapolate the concentration of the detected compounds.

Cell culture conditions

The estrogen receptor positive (ER^+) MCF-7 (ATCC® HTB-22™) and negative (ER^-) MDA-MB-231 (ATCC® HTB-26™) breast epithelial adenocarcinoma cell lines, as well as immortalized normal breast epithelial MCF-10-2A ((ATCC® CRL-10781™) cell lines were obtained from American Type Culture Collection (ATCC;

Manassas, Virginia, USA). Additional ATCC human cells used in this study included; the biphenotypic B myelomonocytic leukemia MV4-11 (CRL-9591™), acute promyelocytic leukemia HL-60 (CCL-240™), chronic myelogenous leukemia K562 (CCL-243™), acute T lymphocytic leukemia Jurkat, (TIB-152™), acute monocytic leukemia THP-1 (TIB-202™) and myeloid histiocytic lymphoma U937 (CRL-1593.2™). MCF-7 cells were cultured in DMEM medium supplemented with 10% (v/v) heat inactivated FBS, 1 mM sodium pyruvate, 1% non-essential amino acids and 10 ng/ml insulin. MDA-MB-231cells were maintained in Leibovitz's L-15 medium containing 10% heat inactivated FBS. The immortalized normal MCF-10-2A breast cell line was grown in DMEM/F-12 having 5% horse serum, 20 ng/ml EGF, 100 ng/ml cholera toxin, 10 ng/ml insulin, and 500 ng/ml hydrocortisone. Hematological cancer cell lines (HL-60, K562, Jurkat, THP-1 and U937), were cultured in RPMI-1640 medium supplemented with 10% heat inactivated FBS, while MV4-11 was grown in IMDM medium. All cell culture media contained penicillin G (100U/mL) and streptomycin (100 mg/mL). Cells were kept at 37°C in a 5% CO2/95% humidified incubator, and passaged when they reached 70% confluency. Cells were seeded at an appropriate density according to each experimental design.

Isolation of peripheral blood mononuclear cells (PB-MNC)

Isolation of (PB-MNCs) from a 15 mL peripheral blood sample of a healthy donor, collected in EDTA tubes, was performed using Lymphoprep™ density gradient medium and 50 ml SepMate™ separation tubes (STEMCELL Technologies Inc., Vancouver, British Colombia, Canada) as described in our recent publication [25] After the separation, PB-MNC were suspended in RPMI-1640 medium at a concentration of $1x10^5$ cells/mL and cultured in 48-well plates for 6 h before treatment with the different concentrations of F-PSEO and L-PSEO.

Cell viability (cytotoxicity) assay

The quantitative determination of viable cells after various treatments with F-PSEO and L-PSEO was performed after staining with the dual DNA intercalating fluorescent dyes kit from EMD Millipore Bioscience (Muse™ Cell Count & Viability Assay) as described in our recent publications [26, 27]. Briefly, 4×10^4 MCF-7, MDA-MB-231 and MCF-10-2A cells/mL, or 3×10^5 cells/mL of each of the hematological cell lines and $1x10^5$ cells/mL of PB-MNCs, were treated with increasing concentrations of F-PSEO or L-PSEO (0.1 to 10.0 µg/mL) for 48 h. Subsequently, samples were incubated with the Cell Count & Viability reagent for 5 min, and the viability of treated cells were analyzed using the flow cytometry-based Muse™ Cell Analyzer (EMD Millipore Bioscience, Darmstadt, Germany) according to the manufacture's protocol. The cell viability was calculated as percentages to live cells compared to untreated control samples containing the carrier solvent DMSO. Under similar conditions, cells were exposed to different concentrations of doxorubicin (1.0 to 10.0 µg/mL), to serve as a positive control for validating of the assay results.

Analysis of apoptosis by flow cytometry

The percentage of MCF-7 and MDA-MB-231 cells undergoing apoptosis after treatment with increasing concentrations of the essential oils was determined by using the Muse™ Annexin-V & Dead Cell Assay kit (EMD Millipore Bioscience, Darmstadt, Germany) according to the manufacturer's protocol. The kit utilizes a fluorescent dye (FITC) conjugated to annexin-V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells and 7-AAD (7-amino-actinomycin D) as dead cells marker. 7-AAD is excluded from living healthy cells, as well as early apoptotic cells. Percentages of cells in early (annexin-V^{+ve}/7-AAD^{-ve}) and late stages of apoptosis (annexin-V^{+ve}/7-AAD^{+ve}) were determined by a flow cytometer-based instrument (Muse™ Cell Analyzer) as previously described [26, 27]. In all experiments, the solvent DMSO concentration was ≤ 0.1%. Similar concentrations of DMSO were added to the control of untreated cells. Both breast cancer cells were also treated with 5.0 µg/mL doxorubicin as an internal positive control to validate the apoptosis results.

Evaluation of nuclear condensation by Hoechst 33342 staining

Breast cancer cells were seeded at a concentration of 1×10^4 cells/mL in an eight-well chamber slide and treated with 0.3 µg/mL of F-PSEO (for both MCF-7 and MDA-MB-231 cells), 2.5 µg/mL and 1.5 µg/mL of L-PSEO (for MCF-7 and MDA-MB-231 cells, respective) or the carrier solvent DMSO (control). After 48 h, the cells were washed with PBS and fixed with 100 µL of 1% glutaraldehyde solution for 30 min. Glutaraldehyde was then removed, and the cells were incubated with 40 µL of the DNA-specific dye Hoechst 33342 (1.0 mg/mL). Morphological changes were observed under Nikon Eclipse 90i® digital fluorescent microscope at a 600X magnification power (Nikon Instruments Inc., Melville, New York, USA).

Measuring caspase-3/-7 enzymatic activities

The enzymatic activities of caspase-3/-7 were determined spectrophotometrically in 20 µg of MCF-7 and MDA-MB-231 lysates after treating the cells with proper concentrations of F-PSEO, L-PSEO or doxorubicin in the presence or absence of 60 µM of the general caspases inhibitor z-VAD-FMK. The assays were performed using the caspase-specific colorimetric tetrapeptide substrate Ac-DEVD-pNA as previously described in details [26-28].

Flow cytometry analysis of cell cycle

MCF-7 and MDA-MB-231 cells were seeded in 6-well plates at a density of 3 x 10^5 cells/mL in their appropriate media and incubated for 12 h. Then, the cells were exposed for 24 h to increased concentrations of F-PSEO and L-PSEO (0.1-5.0 µg/mL). Cells in different stages of cell cycle were analyzed by flow cytometry using the Muse™ Cell Cycle Kit from EMD Millipore Bioscience (Darmstadt, Germany). The kit utilizes propidium iodide (PI) staining to allow quantitative measurements of percentage of cells in the G0/G1, S and G2/M phases on the Muse™ Cell Analyzer (EMD Millipore Bioscience). The cell cycle analysis was performed according to the manufacturer's protocol, and as detailed in our recent reports [27, 29]. Briefly, after incubation with the oils, cells were harvested and washed with PBS, followed by fixation with ice-cold 70% ethanol at -20°C for 3h. Subsequently, cells were washed with PBS, stained with 200 µL of PI/RNAse reagent for 30 min and analyzed by the Muse™ Cell Analyzer. The control cells were treated with equal amount of the carrier solvent (0.1% DMSO), and showed no effect on cell cycle when compared with the solvent-free control cells (not shown). Under similar conditions, cells were treated with 2.0 µg/mL and 5.0 µg/mL doxorubicin as internal controls to validate the assay results (not shown).

Western blot analyses

Sample preparation after treatment with the oils, primary antibodies used in this study and Western blot analysis were performed according to manufacturer's protocols and as previously described in detail [26, 29]. The density of visualized bands was quantitated by UN-SCAN-IT 7.0 gel and graph digitizing software and expressed as a relative-fold to untreated control in two independent experiments.

Statistical analysis

Data presented are the means ± S.D. of results from a minimum of three independent experiments with similar patterns. Statistical analysis was performed using one-way ANOVA and Student's t-test with the aid of GraphPad Prism 6 software. A p value < 0.05 was considered statistically significant.

Results

Composition of the essential oils extracted by hydrodistillation and SPME

The volatile components from flowers and leaves of *P. spinosa* were extracted by hydrodistillation and SPME, and their identities were determined by GC/MS as described under the method section. Representative GC chromatograms for F-PSEO and L-PSEO extracted by hydrodistillation and SPME are shown in Fig. 1A and Fig 1B, respectively. The hydrodistilled flowers and leaves yielded 0.12% and 0.08% of F-PSEO and L-PSEO, respectively. Thirty-eight in F-PSEO and eighty-six compounds in L-PSEO were identified by GC-FID accounting for 99.77%, and 98.69% of the total oils, respectively (Table 1). F-PSEO mostly contained sesquiterpenoids (96.39%), specifically, 78.63% of the oil constituents were oxygenated derivatives. This class of compounds in F-PSEO included acorenone B (33.85%), α-muurolol (21.93%), α-cadinol (8.54%), oplopanonyl acetate (7.22%), δ-cadinene (4.95%) and germacrene-D-4-ol (4.28%). In contrast, L-PSEO was dominated by two groups of compounds: oxygenated sesquiterpenes (51.60%) followed by sesquiterpene hydrocarbons (34.06%). The oxygenated sesquiterpenes [α-cadinol (13.15%), acorenone B (9.64%) and α-muurolol (9.83%)] and the sesquiterpene hydrocarbons [β- longipinene (6.26%) and δ-Cadinene (5.79%)] were the most abundant compounds of these two groups in leaf oil.

On the other hand, GC/MS analysis of the volatile oils extracted by SPME technique identified fifty-nine in F-PSEO and fifty-two compounds is L-PSEO, constituting 97.60% and 98.42% of the total oils respectively (Table 1). The cyclic monoterpenes *p*-cymene (27.76%) and α-phellandrene (13.59%), the sesquiterpene hydrocarbon β-caryophyllene (6.33%),

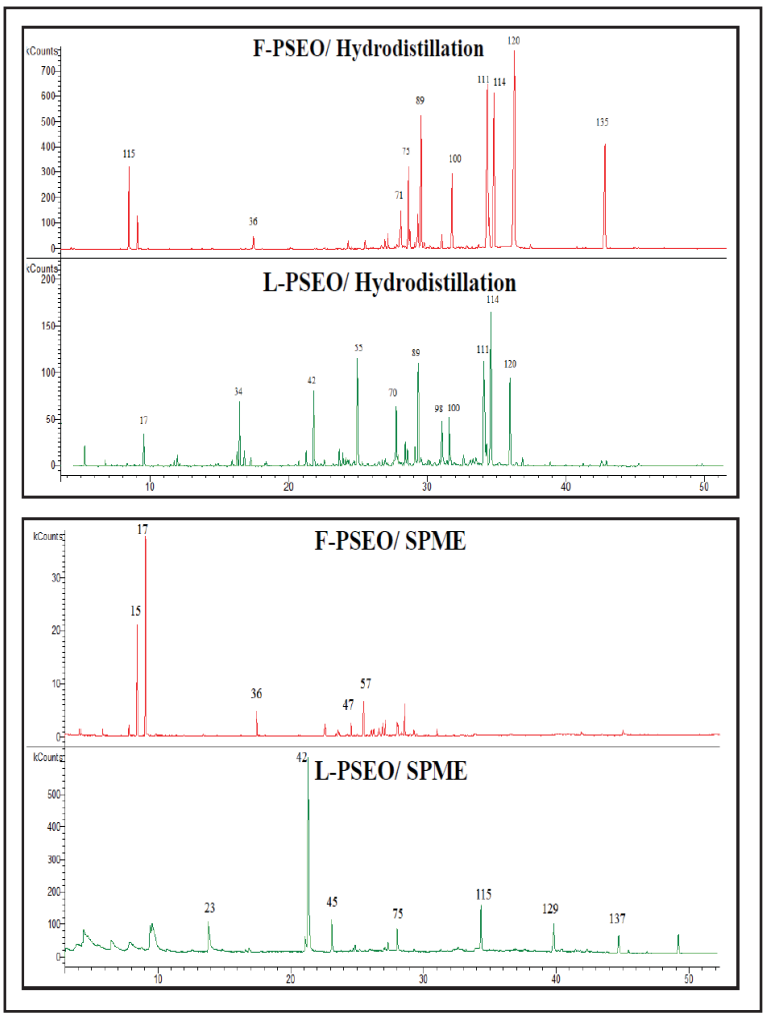

Fig. 1. Representative GC chromatograms obtained for F-PSEO and L-PSEO by hydrodistillation and SPME. The numbers above the peaks indicate the major identified compounds that are listed by the same numbers in Table 1.

the oxygenated monoterpene thymol methyl ether (6.04%) and the sesquiterpene hydrocarbon β-selinene (6.02%) were the major components in F-PSEO. However, the prominent compounds in L-PSEO were the sesquiterpene hydrocarbon persilphiperfol-7-ene (23.84%), the oxygenated monoterpene 1,8-cineole (6.17%) and the monoterpene hydrocarbons [β-pinene (5.84%), *p*-cymene (5.56%), α-pinene (4.91%) and α-terpinene (4.49%)].

In general, there was a compositional and quantitative variation between the oils extracted by SPME and hydrodistillation techniques. The percentage of monoterpene hydrocarbons in SPME-F-PSEO (44.09%) and -L-PSEO (22.27%) samples was greater than the oils extracted by hydrodistillation (3.07% and 0.57%, respectively). The sesquiterpene hydrocarbons and oxygenated monoterpenes were also detected at higher percentage in SPME-F-PSEO (38.64% and 8.82%, respectively) and -L-PSEO (34.15% and 10.61%) as compared to hydrodistilled-flower (17.76% and 0.70%) and -leaf oils (34.06% and 6.66%). Conversely, the hydrodistillation was more effective in extracting oxygenated sesquiterpenes than the SPME method from both flowers (78.63% *vs.*1.53%) and leaves (51.60% *vs.*15.50%) of *P. spinosa.*

Cytotoxicity of F-PSEO and L-PSEO against breast cancer and normal cell lines

Several previous studies have shown that many plant essential oils possess a potent anticancer activity against different cancer cell lines [15, 16]. To investigate the cytotoxic activities of F-PSEO and L-PSEO, the human MCF-7 and MDA-MB-231 cells were exposed to increasing concentrations of the essential oils (0.1-10.0 µg/mL) for 48 h and the viability was determined by flow cytometry after staining with the dual DNA fluorescent dyes as described under the method section. As demonstrated in Fig. 2A and 2B, low doses of F-PSEO and L-PSEO were able to effectively inhibit the growth of both cultured cancer cells in a dose-dependent manner. However, F-PSEO showed much greater cytotoxic effect than L-PSEO. For example, 1.0 µg/mL of F-PSEO and L-PSEO were able to reduce the cellular viability of MCF-7 from 93.5 ± 2.5%, in the untreated control, to 5.5 ± 3.3% and 64.5 ± 4.2%, respectively (Fig. 2A). Similarly, F-PSEO and L-PSEO suppressed the growth of MDA-MB-231 cells from 91.6

± 2.8% (untreated control) to 3.5 ± 2.9% and 47.7 ± 4.1%, respectively (Fig. 2B). Interestingly, MDA-MB-231 cells were more sensitive to exposure to either oil than MCF-7 cells treated under similar conditions. The IC_{50} for F-PSEO and L-PSEO in MDA-MB-231 cells were 0.21 ± 0.03 µg/mL and 1.5 ± 0.10 µg/mL (Fig. 2B), compared to 0.25 ± 0.03 µg/mL and 2.4 ± 0.50 µg/mL in MCF-7 (Fig. 2A), respectively. Interestingly, when compared with the inhibitory doses against the two breast cancer cells, both F-PSEO and L-PSEO showed a much lower toxicity in the noncancerous breast epithelial cells MCF-10-2A grown under similar conditions (Fig. 2C). The IC_{50} for the F-PSEO in MCF-10-2A was at least 5-fold greater than any of the breast cancer cells, while L-PSEO showed only ~1.5 to 2-fold difference, suggesting that the toxicity of the oils is more selective towards malignant cells (Fig. 2C). In contrast, the IC_{50} for the positive control doxorubicin was 4.0 ± 1.0 µg/mL in MCF-7, 1.8 ± 0.5 µg/mL in MDA-MB-231 and 8.3 ± 2.2 µg/mL in MCF-10-2A, indicating a comparatively stronger cytotoxicity and better efficacy exhibited by the oil against cancer cells. The cytotoxicity of both oils was also examined against other cancer cell lines (Table 2). The IC_{50} for F-PSEO and L-PSEO against various hematological cancer cell lines (MV4-11, HL-60, K562, Jurkat, THP-1 and U937) ranged from 0.09 ± 0.02 µg/mL to 0.33 ± 0.08 µg/mL (for F-PSEO) and 0.22 ± 0.05 µg/mL to 1.27 ± 0.31 µg/mL (for L-PSEO), while in PB-MNCs from healthy individuals was 2.80 ± 0.45

Table 1. Chemical composition (%) of F-PSEO and L-PSEO extracted by hydrodistillation and SPME from *P. spinosa*

Compound	RRI[1]	F-PSEO (%)		L-PSEO (%)	
		H-DST[2]	SPME	H-DST	SPME
1-Octene	800	-[3]	0.67	-	-
Ethyl butanoate	809	-	0.55	-	-
2Z-Octene	814	0.1	0.13	-	-
2E-Hexenal	855	-	0.14	-	1.99
3Z-Hexenol	859	-	-	-	2.87
Methyl tiglate	878	-	0.2	-	1.67
Ethyl pentanoate	891	-	0.13	-	-
Nonane	900	-	0.29	-	-
Ethyl tiglate	921	-	1.4	-	-
Alpha-pinene	932	-	0.2	-	4.91
Benzaldehyde	960	-	-	0.2	-
Sabinene	967	-	0.16	-	0.19
β-Pinene	987	-	1.48	-	5.84
Mesitylene	994	-	0.24	-	-
n-Octanal	999	-	0.28	-	-
α- Phellandrene	1007	1.74	13.59	0.36	1.08
α- Terpinene	1018	-	-	-	4.49
p-Cymene	1024	1.33	27.76	0.1	5.56
1,8-Cineole	1030	-	-	0.29	6.17
Benzenacetaldehyde	1041	-	-	1.67	-
E–β-Ocimene	1044	-	0.4	-	-
γ - Tepinene	1054	-	0.14	-	0.2
Isobutyl tiglate	1092	-	-	0.13	
Linalool	1099	-	-	0.33	3.43
cis-Thujone	1104	-	-	0.57	-
2,6-Dimethyl phenol	1109	-	-	0.14	-
1,3,8-Para-menthatriene	1118	-	0.12	-	-
Methyl octanoate	1135	-	0.25	-	0.51
β-Pinene oxide	1159	-	-	-	0.18
Ethyl benzoate	1173	-	-	0.11	
Neoiso-isopulegol	1177	-	0.13	-	-
Iso-pinocampheol	1181	-	0.12	-	-
Safranal	1197	-	-	0.38	-
n-Dodecane	1200	-	0.18	0.85	-
trans-Pulegol	1210	-	-	2.85	-
trans-Carveol	1221	-	0.13	0.74	-
Methyl ether thymol	1228	0.71	6.04	0.41	0.33
Cumin aldehyde	1242	-	0.17	0.1	0.1
Thymol	1289	-	-	0.14	0.4
p-Methoxy acetophenone	1303	-	-	0.31	2.12
z-Patchenol	1321	-	-	0.7	-
Silphiperfol-5-ene	1327	-	-	0.17	-
Persilphiperfol-7-ene	1347	-	2.35	3.17	23.84
δ-Elemene	1330	-	-	0.3	-
Evodone	1340	-	-	0.15	-
α-Cubebene	1350	-	-	0.53	3.82
Cyclsativene	1366	-	0.42		-
Longicyclene	1370	-	1.15	0.71	0.1
α-Ylangene	1373	-	0.91	-	-
z-β-Damascone	1383	-	-	0.86	
β-Elemene	1388	0.17	0.28	0.46	0.21
Sativene	1391	-	-	0.37	-
Z-Jasmone	1392	-	0.16	0.36	-
Methyl perillate	1393	-	2.23	-	-
n-Tetradecane	1401	-	-	0.18	-
β- Longipinene	1404	-	0.29	6.26	-
β-Isocomene	1407	-	0.27		0.18
E-Caryophyllene	1415	0.36	6.33	0.25	0.51
α-trans-Berganotene	1431	-	0.97	-	-
α-Guaiene	1435	-	1.15	0.16	-
Epi-β-Santalene	1444	0.1	1.77	-	-
Z-β-Farnesene	1445	-	-	-	0.27
cis-Muurola-3,5-diene	1450	-		0.14	
α-Himachalene	1451	0.4	2.88		0.79
α-neo-Clovene	1456	0.75	2.87	0.38	-
α-Patxhoulene	1457	-	-	0.62	-
Allo-aromadendrane	1460	-	0.57	0.28	-
9-Epi-(E)-Caryophyllene	1470	0.1	0.19	0.19	2.11
β-Acoradiene	1472	0.17	0.3	0.31	-
Drima-7,9(11)-diene	1475	0.12	0.15		-
trans-Cadina-1(6),4-diene	1476	-	2.69	3.47	-
γ-Muurolene	1481	2.67	1.96	0.81	0.3
Cabreuva oxide D	1482	-	-	0.25	-
α-Amorphene	1485	-	0.59	0.23	0.1
Germactrene D	1487	0.1	0.82	0.13	-
β-Selinene	1491	3.76	6.02	1.45	0.49
δ-Selinene	1493	-	-	0.78	1.05
cis-Cadina-1,4-diene	1496	0.63	0.39	-	-
α-Muurolene	1500	-	-	-	0.17

continued

µg/mL (for F-PSEO) and 3.90 ± 0.36 µg/mL (for L-PSEO), confirming the efficacy of both oils against cancer cells (Table 2).

Compound	RRI[1]	F-PSEO (%)		L-PSEO (%)	
		H-DST[2]	SPME	H-DST	SPME
α-Selinene	1502	-	-	0.1	
Bicyclogermacrene	1503	0.21	0.6	-	-
γ-Patchoulene	1504	-	-	-	-
(E,E)-α-Farnesene	1506	-	0.16	-	-
δ-Amorphene	1509	0.88	1.31	-	-
10-epi-Italicene ether	1510	-	-	-	0.1
γ-Cadinene	1514	1.65	0.62	0.99	0.21
Cubebol	1515	0.12		-	-
Myristicin	1519	-	0.14	-	-
Silphiperfolan-7-beta-ol	1520	0.4	0.14	-	-
δ-Cadinene	1523	4.95	-	5.79	-
Zonarene	1530	0.1	-	0.28	-
trans-Cadina-1(2),4-diene	1533	0.14	0.19	0.1	-
α-Cadinene	1538	-	0.44	-	-
cis-Calamenene	1540	-	-	0.41	-
α-Calacorene	1545	-	-	0.36	-
Selina -3,7(11)-diene	1646	-	-	0.2	-
Silphiperfolan-6- β -ol	1549	-	-	0.16	-
Elemol	1550	0.91	1.39	0.39	-
Germacrene B	1556	-	-	3.51	-
β-Calacorene	1569	-	-	0.91	-
Germacrene-D-4-ol	1579	4.28	-	3.01	0.1
Spathulenol	1578	-	-	0.47	-
Globulol	1583	0.1	-	0.1	-
cis-β-Elemenone	1590	-	-	-	0.71
Viridiflorol	1593	0.1	-	-	-
β-Oplopenone	1603	0.35	-	0.81	-
β-atlantol	1608	-	-	0.18	-
1,10-di-epi-Cubenol	1618	0.13	-	0.55	0.13
β-Cedrene epoxide	1619	-	-	0.71	-
10-epi-gamma-Eudesmol	1624	0.2	-	0.75	-
γ-Eudesmol	1631	-	-	0.11	3.83
α-Muurolol	1644	21.93	-	9.83	2.1
β-Eudesmol	1648	-	-	4.47	-
δ-Cadinol	1650	-	-	1.69	1.45
α-Cadinol	1653	8.54	-	13.15	0.97
Selin-11-en-4-alpha-ol	1661	-	-	-	3.17
Intermedeol	1671	0.1	-	-	-
Cadalene	1676	-	-	0.24	-
Khusinol	1678	-	-	0.21	-
Occidentalol acetate	1681	-	-	0.1	-
Acorenone B	1699	33.85	-	9.64	0.1
Eudesm-7(11)-en-4-ol	1704	-	-	0.74	-
Longifolol	1712	-	-	0.7	-
Guaiol acetate	1722	0.18	-		1.09
z- Nuciferol	1726	-	-	0.14	
(2E,6Z)-Farnesol	1745	-	-	-	0.22
14-Oxy-alpha-Muurolene	1771	0.22	-	-	0.1
2-Hexyl-Z-cinnamaldehyde	1774	-	-	1.75	
14-Hydroxy-alpha-Muurolene	1780	-	-		0.1
Hinesol acetate	1783	-	-	0.52	3.35
n-Octadecane	1802	-	-	0.1	-
Nootkatone	1805	-	-	0.27	-
Drimenone	1809	-	-		0.24
Khusinol acetate	1827	-	-		0.71
(2E,6E)-Farnesyl acetate	1839	-	-	0.27	0.38
Oplopanonyl acetate	1883	7.22	-	0.79	-
Beta-chenopodiol-6-acetate	1891	-	-	0.73	-
Phytol	1927	-	-	0.11	3.38
Total		99.77	97.6	98.69	98.42
Classes of the identified compounds:					
Monoterpenoids Hydrocarbons		3.07	44.09	0.57	22.27
Oxygenated Monoterpenoids		0.71	8.82	6.66	10.61
Sesquiterpene Hydrocarbons		17.76	38.64	34.06	34.15
Oxygenated Sesquiterpenoids		78.63	1.53	51.6	15.5
Oxygenated Diterpene		-	-	0.11	3.38
Aliphatic Compounds		0.1	4.38	2.16	10.39
Aromatic Compounds		-	0.14	2.32	2.12

[1] RRI, Relative Retention Indices on DB-5MS column were calculated from retention times relative to *n*-alkanes and standard reference compounds. [2] H-DST, Hydrodistillation. [3] Absent

The toxicity of F-PSEO and L-PSEO is mediated by apoptosis

Several reports have shown that the anti-proliferative activities of different essential oils towards various cancer cells are mediated by apoptosis [30-32]. To determine whether the cytotoxic effects of F-PSEO and L-PSEO are associated with induction of apoptosis, MCF-7 and MDA-MB-231 cells were treated with increasing concentrations of either oil and then analyzed by flow cytometry after staining with FITC-annexin-V/7-AAD. As shown in Fig. 3, both F-PSEO and L-PSEO induced a dose-dependent increase in the percentage of apoptotic MCF-7 (Fig. 3A) and MDA-MB-231 cancer cells (Fig. 3B). As observed with the viability results, F-PSEO triggered higher percentage of apoptosis in both cultured cells than L-PSEO. Quantitative measurement of the percentages of apoptosis in cultured cells pre-incubated for 48 h with 0.3 and 1.0 µg/mL of F-PSEO were 51.7 ± 6.0% and 71.0 ± 7.2% in MCF-7 cells (Fig. 3A) and 65.0 ± 6.8% and 92.0 ± 5.8% in MDA-MB-231 cells (Fig. 3B), compared to 17.5 ± 3.5% and 33.4 ± 5.4% (Fig. 3A) and 22.5 ± 4.1% and 51.3 ± 4.5% (Fig. 3B) when these cells were exposed to similar concentrations of L-PSEO, respectively. These results also indicated that MDA-MB-231 cells are more sensitive to the treatment with either oil, than MCF-7 cells, and therefore these data are in agreement with those obtained testing the cell viability. Interestingly, there was only a slight marginal variation in the percentages of necrotic cells (annexin-V^-/7AAD$^+$) after exposure to the different concentrations of the essential oils, suggesting that most of the cytotoxic effects of F-PSEO and L-PSEO are mediated by apoptosis. Our finding that inhibition of necrosis by necrostatin-1 did not affect the percentages of dead cells caused by F-PSEO or L-PSEO suggesting that most of the cytotoxic activities of F-PSEO and L-PSEO are mediated by apoptosis

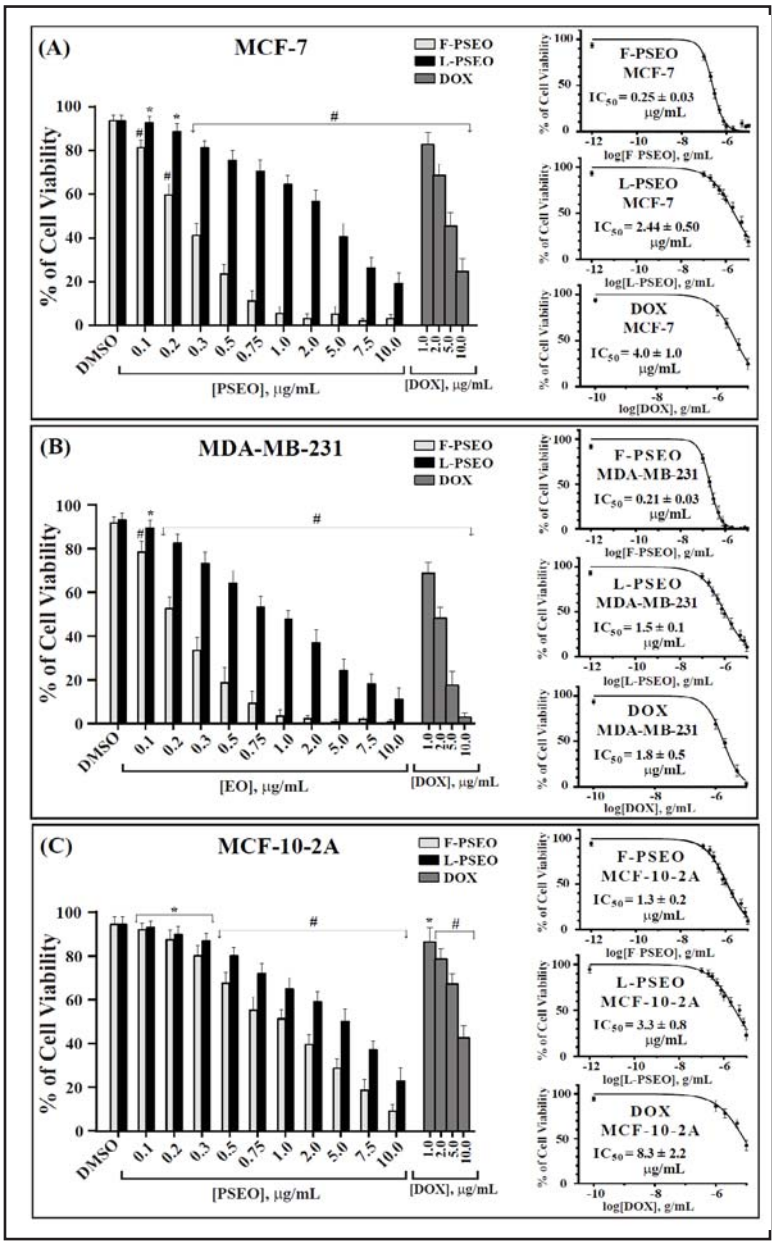

Fig. 2. Low doses of *P. spinosa* essential oils inhibit the growth of breast cancer cell lines in a concentration-dependent manner. (A) MCF-7, (B) MDA-MB-231 breast adenocarcinoma cells and (C) immortalized non-cancerous MCF-10-2A breast epithelial cells were treated with increasing concentrations of F-PSEO and L-PSEO (0.1 to 10.0 µg/mL), or doxorubicin (1.0 to 10.0 µg/mL) for 48 h as indicated in each graph. The percentage of cell viability in each sample was compared to untreated control containing the carrier solvent DMSO. The sigmoidal curves shown were obtained by plotting the mean percentages of viability versus the logarithmic concentrations of the oil in g/mL, and the IC_{50} value was determined using a non-linear regression analysis of GraphPad Prism 6 software. The results shown represent the mean ± SD of three independent experiments. The hash sign (#) was used for values that are significantly different and the astrix (*) for statistically insignificant values as compared to untreated control.

Table 2. The cytotoxicity of *P. spinosa* against different hematological cancer cells and normal PB-MNCs. * Results are expressed as the mean of IC_{50} ± SD of three independent experiments. The IC_{50} value was determined using a non-linear regression analysis of GraphPad Prism 6 software as described in Fig. 2

Cell Line	F-PSEO *IC_{50} (µg/mL)	L-PSEO *IC_{50} (µg/mL)
MV4-11	0.09 ± 0.02	0.22 ± 0.05
HL-60	0.13 ± 0.06	0.35 ± 0.09
K562	0.18 ± 0.07	0.67 ± 0.12
Jurkat	0.22 ± 0.07	0.52 ± 0.10
THP-1	0.24 ± 0.11	0.83 ± 0.16
U937	0.33 ± 0.08	1.27 ± 0.31
PB-MNCs	2.8 ± 0.45	3.9 ± 0.36

(data not shown). Most of the cytotoxicity of the oils in the hematological cell lines were also due to apoptosis (not shown).

Morphological changes induced by F-PSEO and L-PSEO in breast cancer cells

In addition to the increase in the number of annexin-V^{+ve} cells, treatment with the oils was associated with typical morphological changes of apoptotic cells. Both F-PSEO and L-PSEO promoted cellular shrinkage, loss of shape, detachment from adhering surface and condensation/ fragmentation of nuclei in MCF-7 and MDA-MB-231 cultured cells (Fig. 4A

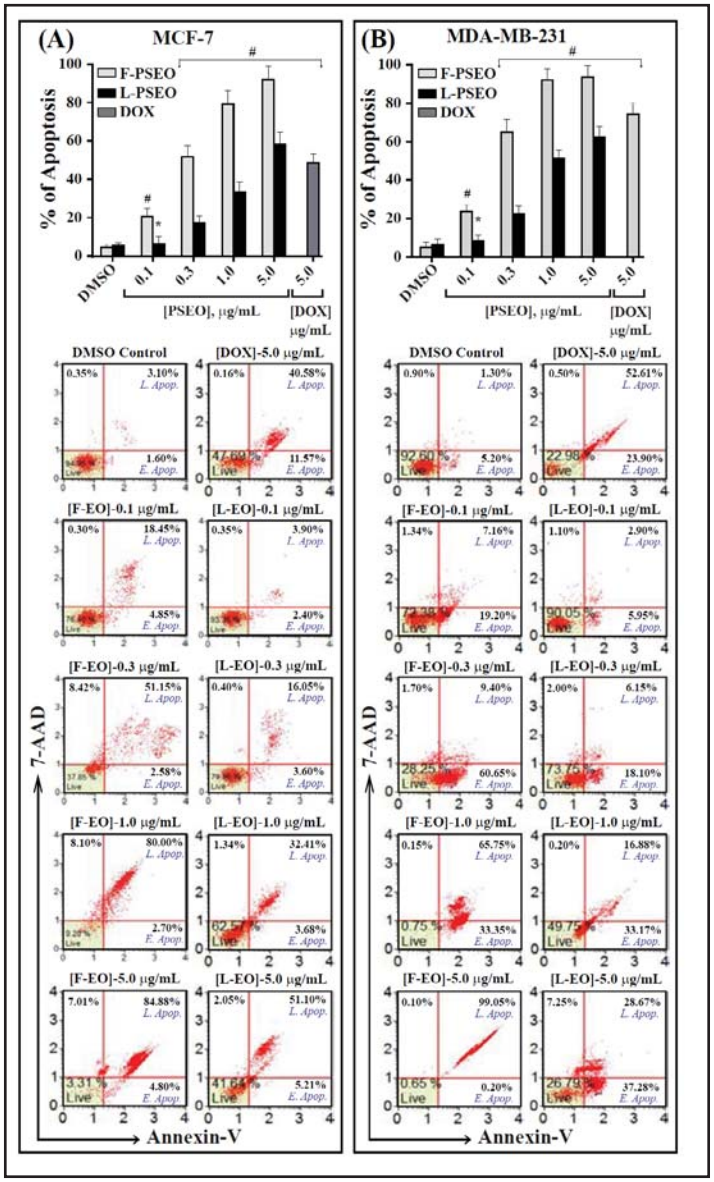

Fig. 3. The cytotoxic effects of *P. spinosa* essential oils against breast cancer cells are mediated by apoptosis. (A) MCF-7 and (B) MDA-MB-231 cells were treated with increasing concentrations of F-PSEO and L-PSEO (0.1 to 5.0 µg/mL) or with 5.0 µg/mL doxorubicin for 48 h and apoptosis was analyzed by a flow cytometer after staining with FITC-annexin-V/7-AAD. The graphs shown represent the summary mean percentages ± SD of apoptosis (early and late apoptosis) of three independent experiments. The scattered flow cytometry blots showing the percentages of early and late apoptosis are indicated for one representative experiment. (#) for values that are significantly different and (*) for statistically insignificant values as compared to untreated control.

and 4B). These changes were not affected by incubating the cells with necrostatin-1 prior to addition of the oils, supporting the fact that they were triggered by apoptosis rather than necrosis (data not shown).

Role of caspases in F-PSEO- and L-PSEO-induced apoptosis

Execution of cellular apoptosis induced by cytotoxic agents including plant extracts requires activation of terminal/executioner caspases, mainly caspase-3 and -7, which are critical for cleaving/inactivation of specific key regulatory proteins [33]. To evaluate the involvement of caspase activation in the essential oil-induced apoptosis, we examined whether treatments with various concentrations of F-PSEO and L-PSEO lead to processing and subsequent activations of caspase-3 in MCF-7 and MDA-MB-232 cells. Processing of caspase-3 was analyzed by Western blotting (Fig. 4C and 4D) and its enzymatic activation, along with caspase-7, by colorimetric assays (Fig.4E and 4F) of lysates obtained from the treated cells. As shown in Fig. 4C and 4D, a dose-dependent processing of caspase-3 was detected by the increased intensity of its p19 cleaved form band after exposure of MCF-7 and MDA-MB-231cells to increasing concentrations of both oils, with maximum levels occurring after exposure to 5.0 µg/mL of F-PSEO and L-PSEO. Processing of caspase-3 paralleled the cleavage of its main substrate PARP. Similar to p19 of caspase-3, the cleaved band of PARP (p97) appeared after treating both cells with 0.1 µg/mL of F-PSEO, while it was only detected after exposing the cells to 0.3 µg/mL of L-PSEO (Fig. 4C and 4D). Thus, processing of caspase-3 and cleavage of PARP coincided with the demonstrated dose-dependent increase in apoptotic cells (Fig. 3) and their morphological changes shown in Fig. 4A and 4B.

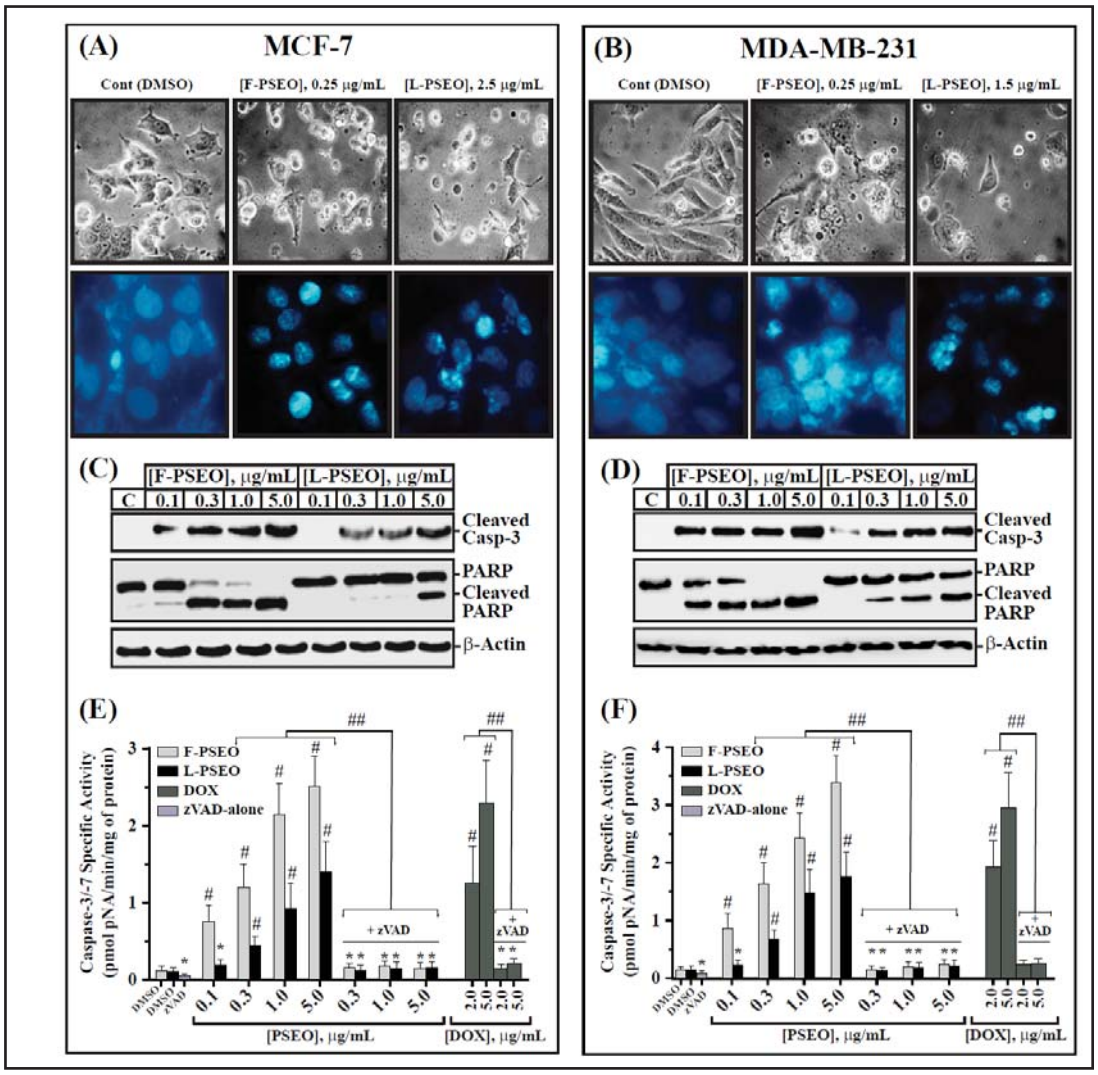

Fig. 4. *P. spinosa* essential oils trigger apoptosis morphological changes and activation of executioner caspases in breast cancer cells. (A) MCF-7 cells were treated with 0.3 µg/mL of F-PSEO or 2.5 µg/mL of L-PSEO and (B) MDA-MB-231 cells with 0.3 µg/mL of F-PSEO or 1.5 µg/mL of L-PSEO for 48 h as indicated on top of each photograph. The cells were photographed before fixation and after staining with Hoechst 33342. The photographs show the morphological changes of unstained cells, and the condensation of chromatin material and fragmentation of nuclei in apoptotic stained cells. (C & D) Western blot analysis showing cleaved form of processed caspase-3 and PARP cleavage in lysates (35 µg) from both cell lines treated with various concentrations of F-PSEO and L-PSEO as indicated on top of each lane. The accurate molecular size of the shown bands was verified by running pre-stained molecular weight standards with each gel. The same membranes were also probed with an antibody against β-actin as loading controls. (E & F) Extracts (20 µg proteins) from MCF-7 and MDA-MB-231 cells treated with different concentrations of F-PSEO, L-PSEO or doxorubicin for 48 h were analyzed for caspase-3/-7 catalytic activities using their specific colorimetric tetrapeptide substrate. The specific enzyme activities, which represent the mean ± SD of three independent experiments, were measured as described in the methods. The cells were also treated with 60 µM of the general caspase inhibitor z-VAD-FMK for 4 h prior to addition of the essential oils or doxorubicin as negative controls. (#) for results that are significantly different and (*) for statistically insignificant values as compared to untreated control.

Detection of the cleaved form of caspase-3 paralleled its activation. As illustrated in Fig. 4E and 4F, 0.1 µg/mL of F-PSEO triggered a significant activation of caspase-3/-7 in both MCF-7 and MDA-MB-231cells, while this activity appeared after treating the cells with 0.3

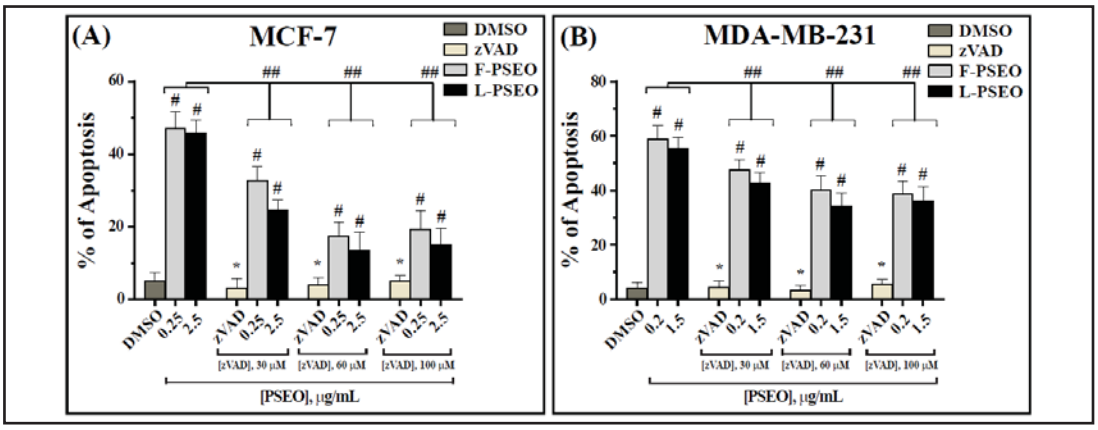

Fig. 5. *P. spinosa* essential oils-induced apoptosis is partially dependent on caspases activation. (A) MCF-7 and (B) MDA-MB-231 cells were exposed to 0.25 µg/mL and 0.20 µg/mL of F-PSEO or 2.5 µg/mL and 1.5 µg/mL of L-PSEO, respectively, in the presence and absence of increasing concentrations of z-VAD-FMK (30 to 100 µM) for 48 h. Subsequently, apoptosis was analyzed after staining with FITC-annexin-V/7AAD as described in Fig. 3. The graphs shown represent the summary of mean percentages ± SD of apoptosis (early and late apoptosis) of three independent experiments. (#) sign, for results that are significantly different and (*) for statistically insignificant values as compared to untreated DMSO control. The double hash sign (##) was used for values that are significantly different from the oil-treated cells in the absence of z-VAD-FMK.

µg/mL of L-PSEO. F-PSEO-induced activation of caspase-3/-7 which reached a mean peak values of 2.52 ± 0.40 and 3.39 ± 0.47 pmol pNA/min/mg protein after incubating MCF-7 and MDA-MB-23 cells with 5.0 µg/mL of the oil, respectively. In contrast, this activity reached only 1.40 ± 0.4 and 1.76 ± 0.43 pmol pNA/min/mg protein after exposing MCF-7 and MDA-MB-23 cells to 5.0 µg/mL of L-PSEO, respectively. In all the tested doses against both cell lines, F-PSEO was able to induce approximately 1.5 to 2.5-fold more activation of caspase-3/-7 than L-PSEO. Again, these results support the findings in Fig. 2 and Fig. 3 that F-PSEO is more toxic to cancer cells than L-PSEO. Under similar conditions, 5.0 µg/mL of doxorubicin triggered a specific caspase-3/-7 activity of 2.30 ± 0.55 pmol pNA/min/mg protein in MCF-7 cells and 2.95 ± 0.61 pmol pNA/min/mg protein in MDA-MB-231 cells, suggesting that both F-PSEO and L-PSEO are comparatively potent inducers of caspase-dependent apoptosis in these cells. The specificity of the detected caspase activity was confirmed by its absence when the cells were pre-incubated with the general caspase inhibitor z-VAD-FMK prior to the addition of the different concentrations of either oil. Furthermore, both oils demonstrated a time-dependent activation of caspase-3/7 in MCF-7 and MDA-MB-231 cells, reaching a maximum activity between 32 and 37 h of incubation (data not shown).

To evaluate the role of caspase activation in the oil-induced apoptosis, MCF-7 and MDA-MB-231 cells were incubated for 6 h with increasing concentrations of z-VAD-FMK (30-100 µM) prior to exposure to a single dose of either F-PSEO (0.25 µg/mL and 0.20 µg/mL) or L-PSEO (2.5 µg/mL and 1.5 µg/mL), corresponding to their approximate IC_{50} in both cell lines, respectively. Subsequently, apoptosis in these samples was analyzed by a flow cytometer after staining with FITC-annexin-V/7AAD. In comparison to MCF-7 cells treated with F-PSEO or L-PSEO alone (Fig. 5A), z-VAD-FMK significantly decreased the oil-induced apoptosis from 47.2 ± 4.5% and 45.8 ± 3.5% to 32.6 ± 4.1% and 24.5 ± 3.0% (with 30.0 µM z-VAD-FMK, corresponding to ~30% and ~47% inhibition, respectively), and to 17.3 ± 4.0% and 13.6 ± 4.8% (with 60.0 µM z-VAD-FMK, corresponding to ~63% and ~70% inhibition, respectively). Importantly, the presence of 60.0 µM of z-VAD-FMK was adequate to completely inhibit the F-PSEO- and L-PSEO-induced activation of the effector caspases illustrated in Fig. 4A. Therefore, further increase in the concentration of z-VAD-FMK to 100 µM did not show additional apoptosis recovery effects in MCF-7 cells (Fig. 5A). These data suggest that the ability of both oils to promote MCF-7 cell death is highly, but not completely, dependent on caspase activation. On the other hand, z-VAD-FMK was less effective in reversing the

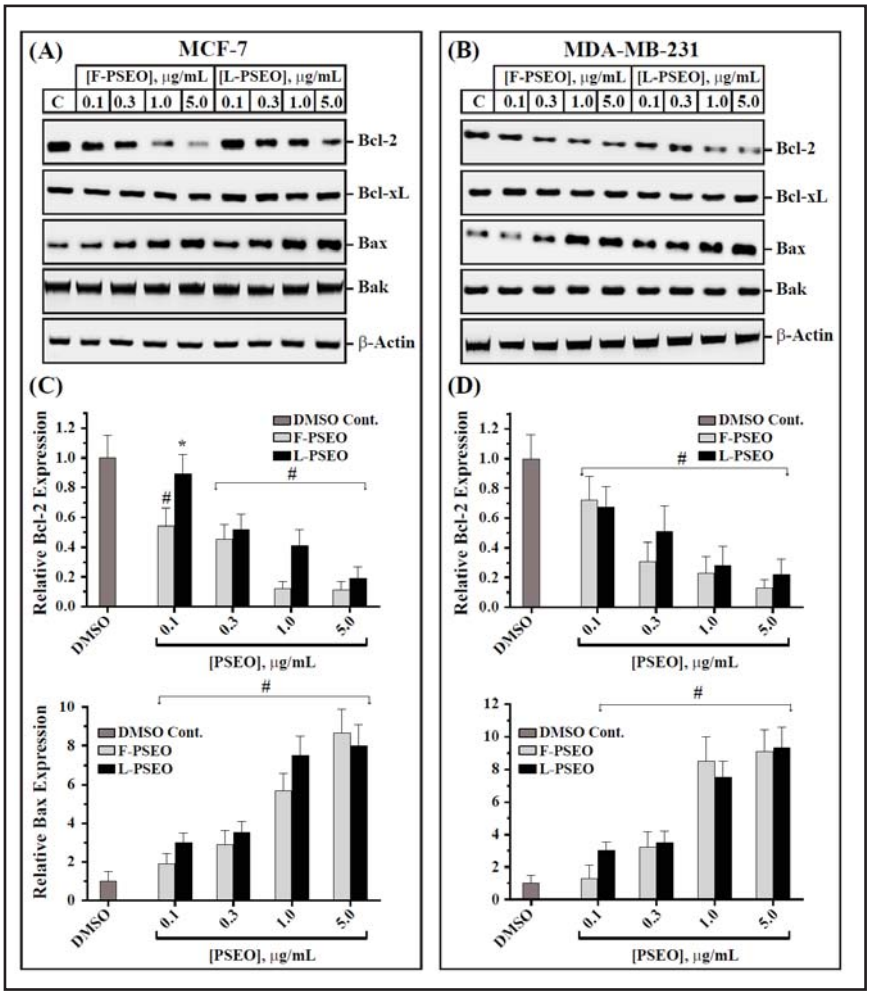

Fig. 6. *P. spinosa* essential oils modulate the expression of anti-apoptotic and pro-apoptotic members of the Bcl-2 family. (A) 35 μg protein lysates from MCF-7 and (B) MDA-MB-231 cells treated with varying concentrations of F-PSEO or L-PSEO were immunoblotted with specific antibodies against Bcl-2, Bcl-X_L, Bax, Bak and β-actin as labeled on each blot. The identity of the shown bands was verified by comparing its corresponding molecular weight to prestained molecular weight standards ran on each gel. (C & D) Densitometric scanning for the intensity levels of Bcl-2 and Bax (relative to untreated control), obtained from Western blotting of the protein in both cell lines treated with different concentrations of the oils for 48 h. The values represent the mean relative band densities ± SD of two independent experiments.

apoptotic effect of F-PSEO and L-PSEO in MDA-MB-231 (Fig. 5B). Pre-incubating cells with 60 ±M z-VAD-FMK caused ~32% (from 58.8 ± 5.1% to 40.2 ± 5.1%) and 38% (from 55.4 ± 4.0% to 34.3 ± 4.7%) suppression of apoptosis induced by F-PSEO and L-PSEO, respectively in the absence of the general caspase inhibitor (Fig. 5B). Thus, the results indicated that the oil induced apoptosis in MDA-MB-231 is partially dependent on caspase activation.

F-PSEP and L-PSEO alter the expression of Bcl-2 and Bax in breast cancer cells

The pro- and anti-apoptotic Bcl-2 family members play a critical role in apoptosis by regulating the translocation of cytochrome c into the cytoplasm to trigger activation of the APAF-1/caspase-9 apoptosome [34]. Our immunoblotting results in Fig. 6 showed that exposure of MCF-7 and MDA-MB-231 to the oils decreased the expression levels of the antiapoptotic protein Bcl-2 and increased the expression of the proapoptotic factor Bax, while the amount of the proapoptotic protein Bak and antiapoptotic Bcl-X_L remain unchanged (Fig. 6A and 6B). Quantification of Bcl-2 revealed that the protein levels gradually decreased with increasing concentrations of the oil, and reached ~11-fold and ~5-fold less than the amount in untreated control after exposure of MCF-7 cells to 5.0 μg/mL of F-PSEO and L-PSEO (Fig. 6C), while the relative amounts of Bax were elevated to ~9-fold and ~8-fold over the control after treatment with an identical concentration of these oils, respectively. Similarly, the protein levels of Bcl-2 were reduced ~8-fold and ~5-fold, and Bax increased ~9-fold as compared to

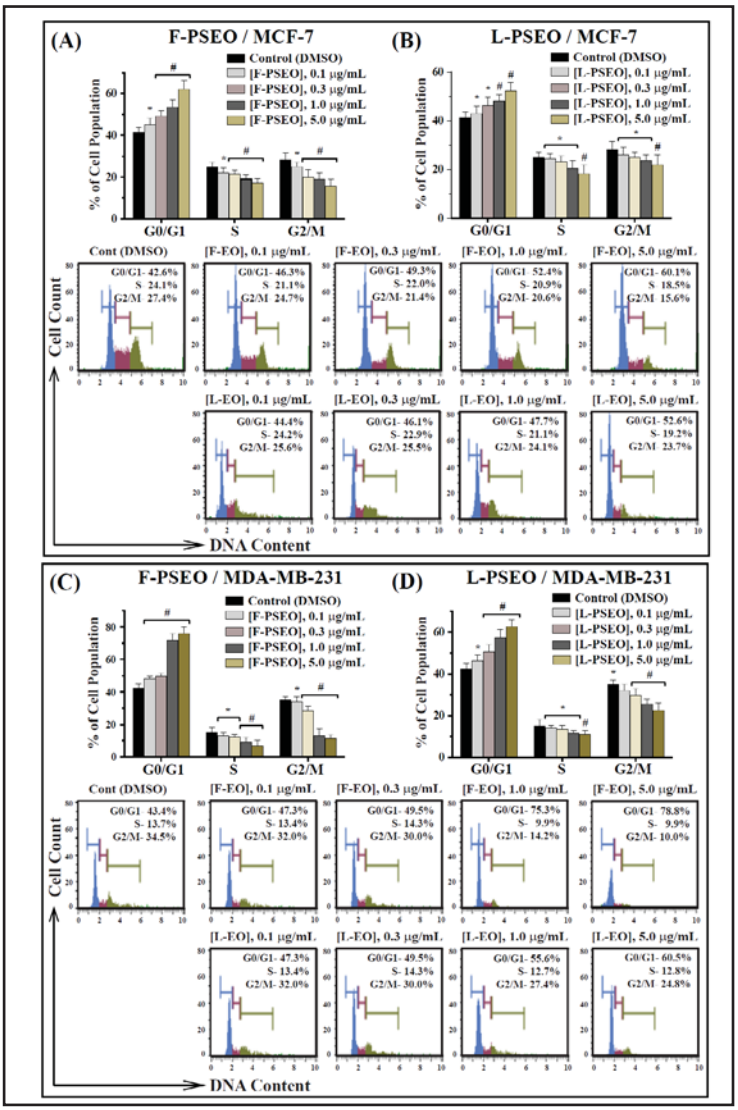

Fig. 7. F-PSEO and L-PSEO induce a dose-dependent cell cycle arrest at G0/G1 phase in MCF-7 and MDA-MB-231cells. (A & B) MCF-7 and (C & D) MDA-MB-231 cells were treated with DMSO (0.1%, control) or increasing concentrations (0.1 to 5.0 µg/mL) of F-PSEO (A & C) and L-PSEO (B & D) for 24 h, and cell cycle analysis was performed as described under the methods. Data shown are representative of three independent experiments. The bar graphs show the mean ± SD of the three independent experiments. The DNA content analysis by flow cytometry is shown for one representative experiment. (#) sign for results that are significantly different and (*) for statistically insignificant values as compared to untreated control.

the protein bands intensity in untreated MDA-MB-231 cells after incubation with 5.0 µg/mL of the respective oils (Fig. 6D). These results strongly suggest that the oil-induced apoptosis involves mitochondrial pathway of apoptosis.

F-PSEO and L-PSEO induce cell cycle arrest in the G0/G1 phase by modulating the expression of regulatory proteins

Several plant essential oils have been documented to arrest cell cycle leading to tumor cell death [35, 36]. When MCF-7 and MDA-MB-231 cells were treated with increasing concentrations of F-PSEO and L-PSEO for 24 h, a dose dependent cell cycle arrest at G0/G1 phase was evident with concomitant decrease in the S and G2/M phases (Fig. 7). However, MDA-MB-231 cells were more susceptible to oil-induced inhibition of cell cycle progression from G1 to S phase than MCF-7 cells. While the percentage of MCF-7 cell population in G0/G1 phase increased from 41.30 ± 2.30% in the absence of the oil to 62.10 ± 4.2% and 52.3 ± 3.6% in the presence of 5.0 µg/mL of F-PSEO (Fig. 7A) and L-PSEO (Fig. 7B), respectively, the increase of the same population in MDA-MB-231 was from 42.4 ± 2.6% (untreated control) to 76.0 ± 4.0% (Fig. 7C) and 62.6 ± 3.4% (Fig. 7D) after treatment with the exact concentration of the respective oils. S-phase cells showed slightly significant decrease, after exposure to 5.0 µg/mL of either oil, but the G2/M cell population dramatically decreased from 35.0 ± 2.1% to 11.4 ± 2.4% (~67% reduction) and 22.5 ± 3.5% (~36% reduction) in MDA-MB-231 treated with F-PSEO and L-PSEO, respectively (Fig. 7C and 7D). In contrast, this population was only decreased from 28.2 ± 3.3% to 15.6 ± 3.5% (~45% reduction, Fig. 7A) and 22.0 ± 4.0% (~22% decrease, Fig. 7B) after the exposure of MCF-7 cells to a similar concentration of F-PSEO and L-PSEO, respectively.

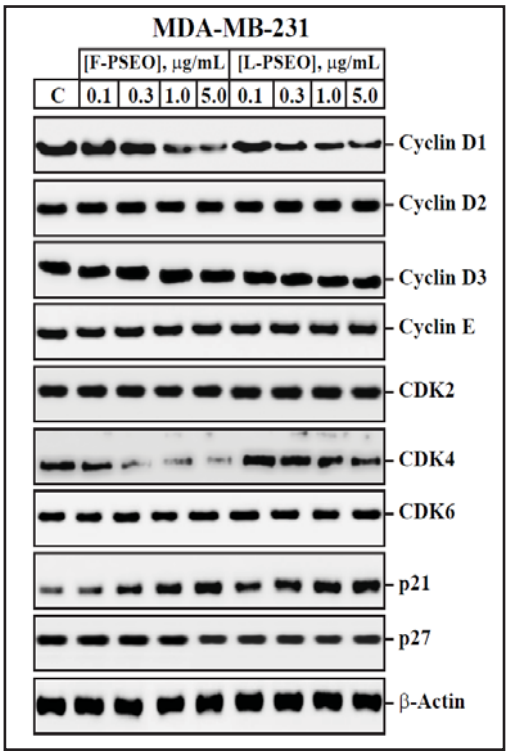

Fig. 8. F-PSEO and L-PSEO modulate the expression of Cyclin D1, CDK4 and p21 in the invasive hormone insensitive breast cancer cell line MDA-MB-231. The cells were treated with 0.1% DMSO (control) or increased concentrations of F-PSEO and L-PSEO (0.1 to 5.0 µg/mL) for 24 h, then 35 µg proteins were analyzed by Western blotting with antibodies against different cyclins (D1, D2, D3 and E), CDKs (2, 4, and 6) and CKI (p21, p27) as labeled on each blot. The immunoblots shown are representations of two independent experiments. The identity of the shown bands was verified by comparing its corresponding molecular weight to pre-stained molecular weight standards ran on each gel. The same membranes were also probed with an antibody against β-actin as loading controls.

Since changes in cell population at different stages was more prominent in MDA-MB-231 treated cells, we analyzed whether these effects were accompanied by alteration in the level of G1 to S transition-regulatory factors. We observed that cyclin D1 along with the cyclin dependent kinase CDK4 were decreased in a dose dependent fashion of the oil, but cyclin D2, cyclin D3, cyclin E, CDK2 and CDK6 were not significantly affected by F-PSEO and L-PSEO treatment (Fig. 8). Expression of the CKI was modulated as exhibited by an increase in p21 level but keeping p27 unchanged (Fig. 8).

Discussion

Each year, more than 1.3 million women worldwide are diagnosed with breast carcinomas. This disease is the leading cause of cancer among women in the developed countries, accounting for about 14% of cancer related deaths and the figures continue to increase. Although the current anticancer drugs continue to play a major role in breast cancer treatment, there are still several subtypes of breast cancer that do not respond effectively to the available therapeutic strategies. Hence, there is an impetus to identify, test and develop more effective treatments [1, 2]. In the current study, we aim to analyze the chemical composition, cytotoxicity and possible modes of action of the essential oil extracted from *Pallines spinosa* flowers and leaves in two different breast tumor cell lines MCF-7 and MDA-MB-231which have two distinct genetic backgrounds that represent most of the reported human mammary carcinomas.

In the last two decades, there has been a substantial focus on natural plant components with potential anticancer activities aiming to develop novel chemotherapeutic agents for cancer [37, 38]. In fact, a substantial number of clinically active drugs that are used in cancer therapy are either natural products or based on natural products, including vinblastine, vincristine, etoposide, teniposide, paclitaxel, doxetaxel, and camptothecin [37]. In line with these studies, we have developed a dedicated research program to identify and characterize components of plants extracts possessing strong anti-proliferative activities. Therefore, the aim of the present study was to identify components of essential oils extracted from the flowers and leaves of *P. spinosa*, examine their anti-proliferative activity and characterize their effect on inducing apoptosis and cell cycle arrest against two different breast cancer cell lines MCF-7 and MDA-MB-231.

In this study, the essential oils were extracted from flowers and leaves of *P. spinosa* by hydrodistillation and SPME to determine their chemical composition by GC and GC-

MS analysis. Our oil yields by hydrodistillation are higher than that reported by Senatore and Bruno [21] from the flower-heads of the same plant collected in Italy. Their chemical investigations show that the flower-heads oil is dominated by oxygenated sesquiterpenes, amounting to 60.2% of the total composition. The main components in this group include germacra-1(10),5-dien-3,4-diol (18.4%), α-cadinol (14.1%), 3-acetoxygermacra-1(10),5-dien-4-ol (13.0%) and T-cadinol (8.2%). In contrast, the oxygenated sesquiterpenes constitute 78.63% of all compounds in our F-PSEO as compared to the previous report [21]. Furthermore, F-PSEO contains acorenone B (33.85%), α-muurolol (21.93%), α-cadinol (8.54%), oplopanonyl acetate (7.22%), δ-cadinene (4.95%) and germacrene-D-4-ol (4.28%) as the major oxygenated sesquiterpenes. The variation in the essential oil content and chemical composition is attributed to well-known factors including geographical location, age of the plant, climate, cultivar and methods of distillation employed [17]. Notably, the comparison between the chemical composition of the leaf and flower oil of *P. spinosa* is only recorded in the present report. L-PSEO contains two groups of compounds: oxygenated sesquiterpenes (51.60%) and sesquiterpene hydrocarbons (34.06%). The oxygenated sesquiterpenes [α-cadinol (13.15%), acorenone B (9.64%) and α-muurolol (9.83%)] and the sesquiterpene hydrocarbons [β- longipinene (6.26%) and δ-Cadinene (5.79%)] were the most abundant compounds of these two groups in leaf oil.

SPME technique is able to extract and concentrate analytes of different volatility, including trace essential oil substances from a very little amount of plant material [17]. In addition, the extraction in this method is performed at low temperatures, hence, the potential thermal degradation of some compounds is negligible when compared to hydrodistillation. Therefore, SPME is preferred over hydrodistillation for detailed GC-MS analytical purposes. In this view, we have compared the composition of the volatile oils extracted by SPME and hydrodistillation. The percentage of monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated monoterpenes are detected at higher concentrations in both F-PSEO and L-PSEO extracted by SPME when compared to hydrodistillation. Conversely, hydrodistilled oils contain more oxygenated sesquiterpenes than that of SPME flower and leaf samples, indicating the high efficiency of hydrodistillation in extracting this group of compounds [17].

Several previous studies have shown that many plant essential oils possess a potent anticancer activity against different cancer cell lines [38-42]. Particularly, plant essential oils from the Asteraceae family are characterized by high contents of cytotoxic oxygenated sesquiterpenes, monoterpenes and monocyclic monoterpenes [43]. Essential oils from members of this family, includes *Inula helenium*, *Inula racemose* and *Inula japonica* which all contain high percentages of the sesquiterpene lactone isoalantolactone [44], and are widely used in Chinese medicine as they have been shown to possess a strong antiproliferative effect against a wide-spectrum of tumor cell lines [44, 45]. Recently, it has been demonstrated that the essential oil from *Inula japonica* sensitizes the multidrug resistant (MDR) MCF-7/ADR cells to doxorubicin induced apoptosis by down-regulating *ABCB1* expression and reducing lipid raft stability [46]. We have also shown that the essential oil from the aerial parts of *Artemisia vulgaris* L. induces apoptosis in the human acute myelogenous leukemia cell line HL-60 by triggering the release of cytochrome c from mitochondria to activate the APAF-1/caspase-9 apoptosome [31]. In addition to HL-60, low doses of *A. vulgaris* volatile oil are able to inhibit the growth of other hematological (Jurkat, K562) and solid tumor (MCF-7, HepG2, PC-3 and HeLa), but lack substantial cytotoxicity against normal immortalized cells such as BJ and HEK-293 V79-4 [31]. Low concentrations of oil from the leaves of *Artemisia herba alba* have been reported to strongly inhibit the growth of P815 mastocytoma and BSR kidney carcinoma cell line, but not normal human peripheral blood mononuclear cells [47]. Moreover, *A. herba alba and A. campestris* are able to kill *Leishmania infantum* promastigotes through induction of apoptosis and halting cells at G0/G1 phase [48]. Furthermore, the sesquiterpenoid hinesol isolated from the essential oil of *Atractylodes lancea* rhizome induces apoptosis through the c-Jun N-terminal kinase (JNK) signaling pathway in HL-60 cells [49].

In line with the above studies, we show here that the essential oils extracted from flowers and leaves of *P. spinosa* (Asteraceae family) exhibit a strong and selective cytotoxic activity against the ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. Importantly, all the tested doses of either oil demonstrate that F-PSEO is a more potent inhibitor of cellular growth than L-PSEO, with IC_{50} values that are ~10-times and ~7-times less than the ones for L-PSEO in MCF-7 and MDA-MB-231 cells, respectively. The differences between the IC_{50} values of F-PSEO and L-PSEO are attributed to both compositional and quantitative variations among these oils. The cyclic monoterpene *p*-cymene, that constitutes a 27.76% (by SPME, 1.33% by hydrodistillation) of the flower oil compared to 5.56% (SPME, 0.1%-hydrodistillation) in L-PSEO, has been reported to exhibit strong antitumor invasive and metastatic actions towards the human fibrosarcoma HT-1080 cells through inhibition of matrix metalloproteinase 9 (MMP-9) along with the suppression of ERK1/2 and p38 MAPK [50]. The cyclic monoterpene α-phellandrene, comprises 13.59% (SPME, 1.74%-hydrodistillation, compared to 1.08% and 0.36% in L-PSEO, respectively), induces apoptosis through the mitochondria-dependent pathway, cell cycle arrest at G0/G1 phase and production of reactive oxygen species (ROS) in murine WEHI-3 leukemic cells [51]. α-phellandrene also alters the expression of cell cycle checkpoint genes such as cyclin G2 and the CDK inhibitor p21, in addition to several apoptosis regulatory factors including caspases [52]. Hsieh LC et al [53] have demonstrated that sub-micromolar concentrations of α-phellandrene promote cell death by autophagy through modulating the expression of mTOR and LC-3II, p53 signaling, and NF-κB activation in J5 liver tumor cells. In addition, the toxicity of this compound involves necrosis when tested in liver cancer cell lines [54]. Furthermore, there is a 12-fold higher percentage of the sesquiterpene hydrocarbon E-caryophyllene in F-PSEO than L-PSEO. This component has been shown to trigger typical morphological changes of apoptosis including nuclear condensation and nucleosomal DNA fragmentation, in addition to inhibition of migration and invasion of colorectal cancer cell lines [55]. Therefore, the more potent anti-proliferative activity of F-PSEO is most probably related to the presence of these strong cytotoxic agents in higher concentrations than the counterparts in L-PSEO, and their potential synergetic actions together with other minor bioactive constituents that are different among the two oils.

On the other hand, the cytotoxicity of the F-PSEO in the non-cancerous epithelial mammary cells MCF-10-2A is ~5 to 6-times less than the one in MCF-7 and MDA-MB-231 cells, suggesting a better efficacy of the oil against tumor cells. The differential effect of F-PSEO between these cell lines may be related to variations in the genetic profile, specific gene expression and distinct cell surface receptors of cancer cells making them more susceptible to the oil [56].

Cell death may occur by many ways such as necrosis, autophagy, mitotic catastrophe, senescence and apoptosis [57]. Apoptosis is the most common and well understood mechanism employed by the cytotoxic agents including plant essential oils [38]. To study the possibility of apoptosis mediated cell death caused by the oils, we have performed a quantitative analysis of apoptosis using Annexin-V/7AAD flow cytometry analysis. Our results show that ~80-90% of MCF-7 and MDA-MB-231 cell population are in early and late stages of apoptosis after exposure to 1.0 µg/mL of F-PSEO, compared to ~30-50% apoptotic cells seen with a similar concentration of L-PSEO. Importantly, necrosis is no greater than 10% under all the tested conditions. Indeed, blocking necrotic cell death with necrostatin-1 did not affect induction of apoptosis by either oil (not shown). In addition, both oils induce typical apoptotic morphological changes in MCF-7 and MDA-MB-231 cells including loss of cellular shape and shrinkage, nuclear condensation and fragmentation. Furthermore, F-PSEO and L-PSEO trigger activation of the effector caspase-3/-7 that typically mediate and execute apoptotic cell death, by cleaving/inactivating essential target proteins required for cell growth and division such as PARP that is evident in our results. Taken together, these findings clearly indicate that both flower and leaf volatile oil of *P. spinosa* induce cell death in MCF-7 and MDA-MB-231 breast carcinoma cells by apoptosis rather than necrosis and, therefore, consistent with the goal of a potential anticancer agent.

Treatment of MCF-7 and MDA-MB-231 cells with F-PSEO or L-PSEO triggers a dose-dependent activation of executioner caspase-3/-7, implying only a mechanism of caspase-mediated cell death. However, our finding that z-VAD-FMK only partially rescued the oil-treated cells from apoptosis suggests the possible involvement of caspase-independent pathways of cell death. This is particularly obvious with the finding that the general caspase-inhibitor is only able to reverse ~30% of the oil induced apoptosis in MDA-MB-231 compared to ~60% in MCF-7 cells. The fact that the essential oils of *P. spinosa* contain compounds that promote cell death by autophagy [53], activation of MAPKs [50], production of ROS [51] and cell cycle arrest [51], proposes that they may also contribute to the cytotoxicity of F-PSEO and L-PSEO.

Apoptosis is triggered by two different mechanisms: one is mediated by the cell surface death receptor (extrinsic pathway), while the other (intrinsic pathway) depends on the release of mitochondrial apoptogenic factors such as cytochrome c and AIF [57]. Although the pathway involved in *P. spinosa* essential oil induced-apoptosis remains to be identified, our results strongly suggest the involvement of the mitochondria in this process. Modulation of pro- and anti-apoptotic protein levels of Bcl-2 family members serves as a key regulator for the release of mitochondrial apoptogenic factors. Pro-apoptotic proteins of the BH3 group, Bax and Bak, inhibit the anti-apoptotic activity of BH4 group proteins, Bcl-2 and Bcl-X_L, by forming heterodimers and exhibit pro-apoptotic activity by forming homodimers [34]. The change in ratio of Bax/Bcl-2 or Bak/ Bcl-x_L causes channel formation in the mitochondrial outer membrane and subsequent release of cytochrome c which eventually leads to caspase-3 activation resulting in cell death *via* apoptosis [34, 58, 59]. Indeed, both F-PSEO and L-PSEO increase the Bax/Bcl-2 ratio by reducing Bcl-2 and elevating Bax levels with increasing doses. This will allow Bax to form the homodimer channels in the outer mitochondrial membrane causing the translocation of cytochrome c to the cytoplasm to promote caspase activation.

Several plant essential oils have been shown to arrest cell cycle, leading to cell death, by modulating the expression or inhibiting the activities of cell cycle regulatory proteins [60]. We thus analyzed the effect of F-PSEO and L-PSEO on cell cycle checkpoint regulation. The lavender essential oil extracted from *Lavandula angustifolia* contains high percentages of linalool and linalyl acetate, which induce apoptosis and the arrest at G2/M phase of cell cycle in PC3 and DU145 prostate cancer cells [61]. Kumar and coworkers [62] have demonstrated that the essential oils from leaves and flowers of *Callistemon citrinus* inhibit cell cycle progression at S and G2/M phases in human lung carcinoma (A549) and rat glioma (C-6) cells. In addition, the Asteraceae *Artemisia campestris* and *Artemisia herba-alba* essential oils effectively kill *Leishmania* promastigotes by apoptosis and halting cell cycle at G0/G1 stage [48]. Here we show that F-PSEO and L-PSEO inhibit cell cycle progression and induces cell-cycle arrest in the G0/G1 phase in MCF-7 and MDA-MB-231 cells in a dose-dependent manner. Similarly to the demonstrated effects as apoptotic inducer, F-PSEO was more powerful in inhibiting cell cycle progression than L-PSEO. Furthermore, MDA-MB-231 cells are found to be more affected by this inhibition than MCF-7 cells. Taken together, these findings indicate that the cytotoxicity of *P. spinosa* essential oils is mediated by apoptosis and cell cycle arrest.

Overexpression of cyclin D1 is associated with the development, progression and metastasis of human mammary carcinomas [63]. Cyclin D1 forms different protein-protein interactions with key cell growth regulators, among which is CDK4, which is also overexpressed in significant number of breast cancer cases [64, 65]. In addition, it has been reported that elevated CDK4 phosphorylation activity is required to maintain the tumorigenesis of breast cancer cells [66]. Therefore, disruption of the functional association between cyclin D1 and CDK4 has been proposed as a target for therapeutic intervention in breast cancer [67]. The major sesquiterpene lactones deoxyelephantopin derived from *Elephantopus scabe* induces apoptosis and inhibits cell cycle progression of HCT116 colorectal cancer cells through downregulating cyclin D1 and CDK4 protein expression [68]. Boswellic acids, extracted from *Boswellia sacra* essential oil have been shown to suppress the expression of cyclin D1, cyclin E, CDK2 and CDK4, as well as inhibit the phosphorylation of retinoblastoma protein RB, and increase production of p21 [69-71]. Consistent with these findings, our results demonstrate

that increased concentrations of either F-PSEO and L-PSEO suppress the expression of cyclin D1 and CDK4 in MDA-MB-231 cells. Therefore, components of *P. spinosa* essential oil, particularly F-PSEO which has a stronger effect on cyclin D1 and CDK4 than L-PSEO, could represent potential chemotherapeutic agents targeting the uncontrolled progression from G1 to S phase in mammary carcinomas.

This study concludes that the selective cytotoxicity of the essential oils extracted from *P. spinosa* flowers and leaves is mediated by apoptosis and cell cycle arrest. The presence of highly active phytochemicals like the cyclic monoterpenes *p*-cymene and α-phellandrene, and the sesquiterpene hydrocarbon E-caryophyllene in these oils may serve as potent natural anticancer compounds to improve human health. The flower oil showed a stronger promising activity on both ER-positive (MCF-7) and ER-negative (MDA-MB-231) cancer cells than the leaf oil, probably due to its higher contents of these bioactive compounds. When compared to the breast cancer cells, the finding that much greater concentrations of both oils are required to inhibit the growth of the non-cancerous mammary cells MCF-10-2A and PB-MNCs suggests their efficacy and warrant further investigations for potential pharmaceutical application.

Ethics Statement

P. spinosa is neither an endangered nor a protected species in Jordan. Cytotoxicity assay using human peripheral blood mononuclear cells (PB-MNCs) was approved by the Institutional Review Board (IRB, under the protocol number RC12/159) of KAIMRC. Written consent as per the standard operating procedure of King Abdulaaziz Medical City was obtained from the volunteer before collection of the blood samples.

Abbreviations

F-PSEO (*Pallines spinosa* flower essential oil); L-PSEO (*Pallines spinosa* leaf essential oil); GC (gas chromatography); GC-MS (gas chromatography-coupled mass spectrometry); GC-FID (gas chromatography–flame ionization detector); SPME (solid phase microextraction); RI (retention index); PB-MNCs (peripheral blood mononuclear cells); Bcl-2 (B-cell lymphoma 2); Bcl-xL (B-cell lymphoma-extra-large); Bax (Bcl-2-associated X protein); Bak (Bcl-2 homologous antagonist killer); PARP (Poly (ADP-ribose) polymerase); CDK (cyclin dependent kinase); CKI (cyclin dependent kinase inhibitor); $p21^{WAF1/CIP1}$ (cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1, simply used here as p21); $p27^{KIP1}$ (cyclin-dependent kinase inhibitor 1B or Kinase inhibitory protein, simply used here as p27); z-VAD-FMK (N-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone); Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide); pNA (p-nitroanilide).

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Disclosure Statement

The authors declare no conflict of interest.

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