Persea declinata (Bl.) Kosterm Bark Crude Extract Induces Apoptosis in MCF-7 Cells via G₀/G₁ Cell Cycle Arrest, Bcl-2/Bax/Bcl-xl Signaling Pathways, and ROS Generation

Putri Narrima,† Mohammadjavad Paydar,† Chung Yeng Looi,† Yi Li Wong,† Hairin Taha,‡ Won Fen Wong,‡ Mustafa Ali Mohd,† and A. Hamid A. Hadi‡

† Department of Pharmacology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
‡ Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

Correspondence should be addressed to Chung Yeng Looi; looicy@um.edu.my

Received 19 November 2013; Accepted 18 February 2014; Published 7 April 2014

1. Introduction

Breast cancer is a heterogeneous disease which is counted as the second leading cause of cancer-related deaths in women worldwide. In recent years breast cancer has become a global public health concern due to the upward trend of its incidence at an annual rate of 3.1%, from 1.38 million women in 2008 to more than 1.6 million in 2010 [1, 2]. In 2007, the number of reported breast cancer cases in Malaysia was 3,242 women, which was 18.1% of the total reported cancer cases and 32.1% of the total cancer cases in women [3]. In 1970, an estrogen receptor-positive cell line, called MCF-7, was derived from a metastatic breast cancer patient at the Michigan Cancer Foundation in 1973 [4], which has become the most extensively used model of estrogen-positive breast cancer cell line for the study of breast cancer as it relates to the susceptibility of the cells to apoptosis [5].

Historically, plants were of the main sources of pharmaceutical agents used in traditional medicine. The application of plant-derived drugs in modern medicine has undergone a dramatic upward trend during the last decades, and a large number of therapeutic compounds (such as vinblastine, taxotere, etoposide, and topotecan) have been discovered in medicinal plants and approved to be used as anticancer drugs [6, 7]. In many countries, medicinal plants are still collected from wild vegetation. But in response to the combined impact of dwindling supplies due to overexploitation of the natural resources and increasing demands by global population growth, medicinal plants are also being cultivated using modern farming systems. Malaysia is rich in biodiversity,
PDM (100 µg/mL)  PDM (50 µg/mL)  PDM (25 µg/mL)  PDM (12.5 µg/mL)  
PDM (6.25 µg/mL)  PDM (3.125 µg/mL)  Negative control (DMSO)  Positive control (2.5 µg/mL doxorubicin)

(a)

(b)

Figure 1: PDM induced significant reduction of cell number, cell shrinkage, and apoptotic body formation in treated MCF-7 cells. (a) MCF-7 cells were treated with DMSO, doxorubicin, or different concentrations of PDM for 24 h. Live cell images indicating cell shrinkage and apoptosis in a dose-dependent manner. (b) MCF-7 cells were treated with DMSO or PDM (25 µg/mL) and were screened for 24 h using a real-time cellular analysis (JuLi Br) system. Real-time cell proliferation throughout the 24 hours of treatment indicating significant reduction of cell number, cell shrinkage, and formation of apoptotic body in the MCF-7 cells treated with PDM.

which is believed to be 130 million years old, and is mostly covered with enormous forests that include an estimated 14,500 species of flowering plants. About 15% of these plants were claimed to have medicinal properties, of which only a handful have been studied for their potential bioactivities and are currently cultivated by various farming communities, but there are still many more plants to be discovered [8].

Persea declinata (Bl.) Kosterm (common names: medang inai, medang tanah, kayu helah, huru manok, huru leu-ur, and meang telut) is a member of the Lauraceae family, which is widely distributed in Borneo, Java, Malaysia (Penang, Kelantan, Terengganu, Pahang, Selangor) and Singapore. It is an evergreen tree which can grow above 30 m tall, with smooth gray bark. The leaves are elliptic to lanceolate (long: from 5.5 to 13 cm, width: 2–3.5 cm), subcoriaces, cuneiform at the base, the apex acute, and the petiole glabrous (long: 5–15 mm) with globular fruits (diameter: 5–10 mm). It shares the same genus with the popularly investigated Persea sp., avocado (Persea americana Mill). Avocado is widely consumed as food and for medicinal purposes. It possesses various potential cancer preventive phytochemicals [9] and other pharmacological properties including hepatoprotective [10], anticonvulsant [11], wound healing [12], analgesic, anti-inflammatory [13], hypoglycaemic, and hypocholesterolaemic [14].

While countless studies have been done on avocado, Persea declinata (Bl.) Kosterm on the other hand has never been investigated for any pharmacological potential. Therefore, the present study will focus on the preliminary cytotoxic testing of Persea declinata (Bl.) Kosterm bark methanolic crude extract on various cancer cell lines and the effective one is used as a model to further investigate the mechanistic action.

2. Materials and Methods

2.1. Chemical Reagents and Solvents. Chemical reagents and solvents used for extraction and assays were of analytical grade and were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Plant Source. The bark of Persea declinata (Bl.) Kosterm was collected from Dungun, Terengganu, Malaysia. The plant species was identified by the university’s botanist with a Voucher specimen (no. KL 5068) and was deposited in the herbarium of the Chemistry Department, University of Malaya.

2.3. Cell Culture. Human colon adenocarcinoma cell line (HT29), human hepatocarcinoma cell line (HepG2), human
breast cancer cell lines (T47D, MDA-MB-231), and human normal hepatic cell line (WRL-68) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Human breast cancer cell line (MCF-7) was purchased from Cell Line Services (300273; Eppelheim, Germany), and oral carcinoma cell lines (H400, H413, and BICR31) were provided by Professor Ian Charles Paterson. HT29, HepG2, T47D, MDA-MB-231, and MCF-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 1% penicillin and streptomycin. WRL-68 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc., Rockville, MD) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 1% penicillin and streptomycin. H400, H413, and BICR31 cells were grown in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 1% penicillin and streptomycin. Cells were cultured in tissue culture flasks (Corning, USA) and were kept in an incubator at 37°C in a humidified atmosphere with 5% CO2. For experimental purposes, cells in exponential growth phase (approximately 70–80% confluency) were used.

2.4. Extraction. 50 grams of grounded bark of Persea declinata (Bl.) Kosterm was extracted with 500 mL of methanol. Approximately 5 g of polyvinylpyrrolidone (PVP, Sigma-Aldrich, USA) was added for detannification and the mixture was kept for 48 hours. After that, the crude extract (PDM) was filtered and evaporated to about 15 mL volume using a rotary evaporator and further frozen and freeze-dried for another 48 hours.

2.5. MTT Cell Viability Assay. The cytotoxic effect of PDM was assessed by MTT cell viability assay against different cancer cells [19]. 1.0 x 10^4 cells were seeded in a 96-well plate and incubated overnight at 37°C in 5% CO2. On the next day, the cells were treated with a two-fold dilution series of six concentrations of PDM, and then they were incubated at 37°C in 5% CO2 for 48 hours. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) solution was added at 2 mg/mL and after 2 hours of incubation at 37°C in 5% CO2, DMSO was added to dissolve the formazan crystals. The plates were then read at 570 nm absorbance. The cell viability percentage after exposure to PDM for 48 hours was calculated by a previously described method [20]. The ratio of the absorbance of treated cells to the absorbance of DMSO-treated control cells was determined as percentage of cell viability. IC50 value was defined as the concentration of PDM required to reduce the absorbance of treated cells to 50% of the DMSO-treated control cells. The experiment was carried out in triplicates.

2.6. Real-Time Cell Proliferation. In vitro proliferation of PDM-treated and untreated cells was surveyed using a Real-Time Cellular Analysis (JuLi Br) system. 2.0 x 10^5 cells were seeded in a 6-well plate and incubated overnight at 37°C in 5% CO2. The RTCA system monitored the proliferation of cells every 5 minutes for about 24 hours. During the log growth phase, the cells were treated with IC50 concentrations of the PDM (17 μg/mL) or left untreated and monitored continuously for another 24 hours.

2.7. LDH Release Assay. Measurement of lactate dehydrogenase (LDH) release is a biomarker that can determine the cytotoxicity of a compound or an extract. Briefly, MCF-7 cells were treated with different concentrations of PDM and Triton X-100 (positive control) for 24 h, and the supernatants of the untreated and treated cells were transferred to a new 96-well plate for LDH activity analysis. Next, 100 μL of LDH reaction solution was added to each well, the plate was incubated at room temperature for 30 min, and the absorbance was read at 490 nm using a Tecan Infinite 200 Pro (Tecan, Männedorf, Switzerland) microplate reader. The amount of formazan salt and the intensity of red color in treated and untreated samples were represented as the LDH activity of cells.

2.8. Cell Cycle Analysis. 1 x 10^4 cells per well were seeded in a 96-well plate and incubated overnight at 37°C in 5% CO2. Cells were treated with different concentrations of the PDM or DMSO (negative control) for 24 hours. BrdU and Phospho-histone H3 dyes were added into live cells for 30 minutes. Cells were fixed and stained as described by the manufacturer's instruction. Stained cells were visualized and acquired using Cellomics ArrayScan HCS reader (Thermo Scientific). Target activation bioapplication module was used to quantify the fluorescence intensities of dyes.

2.9. Reactive Oxygen Species (ROS) Assays. 1 x 10^4 cells per well were seeded onto a 96-well plate. Cells were treated with the PDM or DMSO (negative control) at indicated concentrations for 12 hours. Dihydroethidium (DHE) dye contained in Cellomics ROS kit was added into a live...
Figure 3: (a) Effect of PDM on cell cycle arrest. The MCF-7 cells were treated with DMSO, doxorubicin, or PDM for 24 h, stained with BrdU and Phospho-histone H3, and subjected to the Cellomics ArrayScan HCS reader for cell cycle analysis. (b) Representative bar charts indicating that PDM treatment caused no significant changes in BrdU or Phospho-histone H3 fluorescence intensities, and consequently induced no cell cycle arrest in the treated MCF-7 cells. Data were expressed as the mean ± SD of fluorescence intensity readings for three independent experiments. (c) Cells were stained with Hoechst and the distribution in G0/G1, S, and G2/M cell cycle phases was determined with Cellomics High Content Screening.
culture for 30 minutes. Cells were fixed and washed with wash buffer as described by the manufacturer’s instruction. Stained cells were visualized and acquired using Cellomics ArrayScan HCS reader (Thermo Scientific). Target activation bioapplication module was used to quantify the fluorescence intensities of DHE dye in the nucleus.

2.10. Nuclear Morphology, Membrane Permeability, and Mitochondrial Membrane Potential (MMP) Assays. Cellomics multiparameter cytotoxicity 3 kit (Thermo Scientific) was used as described previously [21]. $1 \times 10^4$ cells per well were plated in a 96-well plate and incubated overnight at 37°C in 5% CO₂. The cells were treated with different concentrations of the PDM and further incubated at 37°C in 5% CO₂ for 24 hours. MMP dye and the cell permeability dye were added to live cells and incubated for 1 hour. After fixing the cells, the nucleus was stained with Hoechst 33258. Stained cells were visualized and images were captured using a camera connected to a fluorescent microscope.

2.11. DNA Fragmentation Analysis by Acridine Orange. $1 \times 10^4$ cells per well were plated in a 96-well plate and incubated overnight at 37°C in 5% CO₂. PDM at various concentrations were added and further incubated for 24 hours. Acridine orange staining solution was added to live cells and incubated for 15 min. The cells were then fixed and visualized and images were captured using a camera connected to a fluorescent microscope.

2.12. Bioluminescent Assays for Caspase-3/7 Activities. A dose-dependent study of caspase-3/7 activity was performed in triplicates using assay kits Caspase-Glo 3/7 (Promega, Madison, WI) on a white 96-well microplate. A total of $1 \times 10^4$ cells were seeded per well and incubated with different concentrations of PDM for 24 hours. Caspase activities were investigated according to the manufacturer’s protocol. Briefly, 100 μL caspase-Glo reagent was added and incubated at room temperature for 30 minutes. Activated caspases cleaved the aminoluciferin-labeled synthetic tetrapeptide, leading to release of luciferase substrate. The caspase activities were measured using a Tecan 12 Infinite 200 Pro (Tecan, Männedorf, Switzerland) microplate reader.

2.13. NF-κB Translocation. Briefly, $1.0 \times 10^4$ cells were seeded in a 96-well plate and incubated overnight at 37°C in 5% CO₂. The cells were pretreated with different concentrations of PDM for 3 h and then stimulated with 1 ng/mL TNF-α for 30 min. The medium was removed and the cells were fixed and stained with Cellomics nucleus factor-κB (NF-κB) activation kit from Thermo Scientific based on the manufacturer’s instructions. The plate was evaluated on Array Scan HCS Reader. The calculation of cytoplasmic and nuclear
NF-κB intensity ratio was carried out using Cytoplasm to Nucleus Translocation BioApplication software. The average intensity of 200 objects (cells) per well was quantified. The ratios were then compared among TNF-α-stimulated, treated, and untreated cells [21].

2.14. Western Blot Analysis. SDS-PAGE and Western blot analyses were done as described with slight modifications [22]. Briefly, 24 hours posttreatment, cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with freshly added 10 mM β-glycerophosphate,
Untreated PDM 25 𝜇g/mL

Acridine orange average fluorescent intensity

| Concentration (µg/mL) | Acridine orange average fluorescent intensity |
|-----------------------|----------------------------------------------|
| Control               | 6.25                                         |
| 6.25                  | 12.5                                         |
| 12.5                  | 25                                           |
| 25                    | 50                                           |
| 50                    |                                              |

Figure 6: (a) Acridine orange DNA fragmentation analysis. The cells were treated with DMEM or PDM for 24 h and stained with acridine orange dye. (b) Representative bar charts that show dose-dependent increased fluorescent intensity of acridine orange dye inside the nucleus of the PDM-treated MCF-7 cells, indicating DNA fragmentation.

Fold higher than control

![Caspase 3/7](image)

Figure 7: Relative luminescence expression of caspases 3/7 in MCF-7 cells treated with various concentrations of PDM.

Table 1: IDs for TaqMan gene expression assays.

| Target gene | Assay ID          |
|-------------|-------------------|
| GAPDH       | Hs02758991_g1     |
| Bcl-2       | Hs00608023_ml     |
| Bcl-xl      | Hs00236329_ml     |
| Bax         | Hs00180269_ml     |

Table performed with rabbit anti-Bcl-2, anti-Bcl-xl, and anti-Bax antibodies (1:200) (Cell Signaling Technology, Danvers, MA). Membranes were washed 3 times (10 min each) in Tween buffer before incubating with HRP-conjugated goat anti-mouse or rabbit secondary antibodies. To remove excess antibodies, membranes were washed 4 times before HRP activities were detected using ECL Plus Chemiluminescence Reagent (Amersham, Chalfont, UK) according to the protocol supplied with the kit.

2.15. Quantitative PCR Analysis. MCF-7 cells were treated with various concentrations of extract for 24 h. Total RNAs were isolated with Zymo Research Quick-RNA MiniPrep kit. Complimentary DNAs were synthesized with Applied Biosystems High Capacity RNA-to-cDNA Kit. Quantitative PCR was performed with Applied Biosystems TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assays and run on a real-time PCR machine (Applied Biosystems StepOnePlus system). All data were then normalized to GAPDH. The IDs for TaqMan Gene Expression Assays used in this experiment are listed in Table 1.
2.16. GC-TOFMS Identification and Chemical Analysis of Crude Extract. Gas chromatography time of flight mass spectrometry (GC-TOFMS) analysis of the crude extract was carried out on a Pegasus HT GC-TOFMS 7890A (LECO, USA) system. Separation was conducted on an RXI-5 MS column (30 m × 0.32 mm × 0.25 μm), with helium as the carrier gas (flow rate of 1.0 mL/min). The injection volume was 1 μL in a split mode. The column temperature was initially held at 40°C for 5 minutes and then increased to 260°C at a rate of 10°C/min, then maintained at 260°C for 10 minutes. The temperatures of the injector and detector were 250°C and 280°C, respectively. Mass acquisition was performed in the range of 40–550 atomic mass units (a. m. u) using electron impact ionization at 70 eV. The major components in this sample were predicted by a spectral database matching against the library of National Institute of Standards and Technology (NIST21 and NISTWiley).

2.17. Statistical Analysis. Experimental values were presented as the means ± standard deviation (SD) of the number of experiments indicated in the legends. Analysis of variance (ANOVA) was performed using GraphPad Prism 5 software. Statistical significance was defined when P < 0.05.

3. Results

3.1. Effect of PDM on Cell Viability. The cytotoxic effect of PDM was evaluated on HepG2, MDA-MB-231, MCF-7, T47D, H400, H413, BICR31, and WRL-68 cells using MTT assays. Table 2 shows the IC_{50} values after 48 hours of treatment with PDM. Highest cytotoxicity was observed in MCF-7 breast cancer cells (IC_{50} = 16.68 ± 0.89). Moreover, PDM exhibited higher selectivity on human breast cancer MCF-7 cells, compared to human normal hepatic WRL-68 cells.

Next, real-time cell proliferation assay was carried out to monitor the morphological changes of MCF-7 cells treated with PDM. The results indicated significant reduction of cell number, cell shrinkage, and apoptotic body formation throughout the 24 hours of treatment (Figure 1).

3.2. PDM Induced Higher LDH Release in MCF-7 Cells. Lactate dehydrogenase (LDH) release in the medium is a marker that shows the loss of membrane integrity, apoptosis, or necrosis. The cytotoxicity of PDM, as determined by the LDH release assay, was quantified on MCF-7 cells treated with various concentrations of the extract for 24 h. PDM induced
a significant elevation in LDH release, demonstrating cytotoxicity at 25 and 50 μg/mL concentrations compared to the control cells (Figure 2).

3.3. Effect of PDM on Cell Cycle. We investigated the cell cycle arrest of MCF-7 cells treated with different concentrations of PDM, using BrdU and Phospho-histone H3 dyes. Comparison of the images and intensity bar charts of PDM-treated and untreated MCF-7 cells indicated a decreased level of BrdU and Phospho-histone H3 intensities (Figures 3(a) and 3(b)). The histogram plot on Hoechst total intensity also demonstrated a decreased level of cells in S and G2/M phases and an increased number of cells at G0/G1 in PDM-treated MCF-7 cells (Figure 3(c)). These results show that PDM induced G0/G1 arrest in MCF-7 after 24 hours.

3.4. PDM Increased Reactive Oxygen Species (ROS) Production. ROS is produced as a byproduct of normal metabolism
of oxygen. ROS formation may undergo a drastic increase under environmental or chemical stress. Enhanced levels of ROS may lead to apoptosis or cell cycle arrest. In this study, we stained PDM-treated (24 hours) or untreated MCF-7 live cells with DHE dye to assess whether the exposure of PDM promotes ROS production. As shown in Figure 4, the levels of DHE increased significantly in MCF-7 cells treated with PDM, indicating higher ROS production.

3.5. PDM Decreased Mitochondrial Membrane Potential (MMP) and Increased Cell Membrane Permeability. As the main source of cellular ROS and adenosine triphosphate
Table 2: Effect of *Persea declinata* (Bl.) Kosterm bark crude extract on cells expressed as IC<sub>50</sub> values in 48 hours MTT assay.

| Cell line       | IC<sub>50</sub> (μg/mL) |
|-----------------|-------------------------|
| Bicr31          | 27.68 ± 2.14            |
| H400            | 31.39 ± 1.85            |
| H413            | 24.16 ± 1.44            |
| MCF7            | 16.68 ± 0.89            |
| T47D            | 18.53 ± 1.26            |
| MDA-MB-231      | 21.80 ± 1.71            |
| HepG2           | 26.41 ± 2.09            |
| HT-29           | 28.59 ± 1.93            |
| WRL-68          | 97.90 ± 5.73            |

3.6. PDM Induced DNA Fragmentation. Acridine orange (AO) is used to determine DNA integrity. We stained MCF-7 16 cells with acridine orange dye after 24 hours of treatment. Figures 6(a) and 6(b) showed a dose-dependent increase of nucleus restricted acridine orange dye in PDM-treated cells, indicating DNA fragmentation.

3.7. PDM Increased Caspase-3/7 Activity. The excessive production of ROS from mitochondria and the collapse of MMP may activate downstream caspase molecules and consequently lead to apoptotic cell death. To examine this, we measured the caspase-3/7 activity using bioluminescent assays. As shown in Figure 7, a significant dose-dependent increase in caspase-3/7 activity was detected in PDM-treated cells. Hence, the apoptosis induced by PDM in MCF-7 cells could be mediated through caspase activation.

3.8. Effect of PDM on NF-κB Activation. The nuclear factor kappa B (NF-κB) is a transcription factor, critical for cell proliferation and apoptosis. Activation of NF-κB is indicated by cytoplasm to nuclear translocation to enable DNA-binding activity and facilitate target gene expression. NF-κB remains in the cytoplasm in the absence of activation signal in MCF-7 (Figure 8(a)). In the presence of TNF-α, NF-κB localized mainly in the nucleus of most cells (Figure 8(a)). As shown in Figure 10, PDM treatment has no inhibitory effect on TNF-α-induced NF-κB translocation from cytoplasm to nucleus, compared to positive control curcumin (Figures 8(a) and 8(b)).

4. Discussion

Fruits and vegetables contain multiple anticancer phytochemicals, which have been extensively explored as a cancer prevention approach. Studies have shown that avocado fruit extracts exhibited antiproliferative effects in human cancer cell lines [23]. By studying the same avocado family, we found that PDM is active on various cancer cells, demonstrating...
Table 3: Compounds tentatively identified in *Persea declinata* (BL) Kosterm bark crude extract.

| Peak number | RTa (min) | Percentage of the peakb | Molecular weight | Molecular formula | Similarity index | Compound |
|-------------|-----------|-------------------------|------------------|-------------------|------------------|----------|
| 1           | 17.025    | 10.226                  | 204              | C₁₅H₂₄             | 89.1             | α-Copaene [15] |
| 2           | 17.643    | 21.877                  | 204              | C₁₅H₂₄             | 90.6             | Caryophyllene [16] |
| 3           | 18.082    | 5.996                   | 204              | C₁₅H₂₄             | 92.2             | Iso-α-humulene [17] |
| 4           | 31.123    | 61.901                  | 166              | C₁₀H₁₄O₂            | 87.3             | 2-Methoxy-4-propylphenol (4-propylguaiacol) [18] |

Total 100.04

aRT: retention time (min).
bPeak area relative to the total peak area percentage.

Excessive production of ROS, known as oxidative stress, may cause cell death by nonphysiological (necrotic) or regulated pathways (apoptotic). Previous studies indicated that death receptors, including the TNF receptor-1 (TNF-R1), are able to initiate caspase-independent cell death. This form of necrotic cell death appears to be dependent on the generation of ROS, through activation of poly(ADP-ribose) polymerase (PARP) [26, 27]. In this study, we observed a dose-dependent increase in ROS level after PDM treatment. PDM-induced ROS production could affect mitochondria’s function, which has been shown to play an indispensable role in cell survival.

The decrease of MMP fluorescent intensity and the increase in cell membrane permeability in PDM-treated cells might be due to excessive generation of ROS.

Increased level of ROS and MMP collapse may also activate cysteine proteases (caspases) which converged on caspase 3/7. Caspase 3/7 normally cleaves several target proteins such as PARP (the enzyme responsible for repairing DNA) and leads to apoptotic DNA fragmentation. Using acridine orange dye, DNA fragmentation and cleavage were detected in PDM-treated MCF-7 cells. These results suggest that PDM induced apoptosis via caspase 3/7 activation. Since MCF-7 is a cell line deficient in caspase-3 expression, it is possible that DNA fragmentation could be mediated by activation of caspase-7 and PARP cleavage, as shown previously by another study [28].

Analysis of volatile nonpolar constituents of PDM by GC-TOFMS showed 4 major compounds, which exhibit antitumor potential for further development. In fact, PDM exhibited higher selectivity on MCF-7 breast cancer cells and was less cytotoxic towards WRL-68 normal hepatic cells. This is a good indication as some compounds have been shown to have side effects such as causing liver or kidney toxicity [24, 25].
various bioactive evidence. The sesquiterpene hydrocarbon α-copaene has been shown to exhibit antibacterial activities [29–31], whereas, α-humulene was discovered to be cytotoxic against MCF-7 cancer cells [32, 33]. Another study showed that this compound was active against human lung carcinoma A-549 and colon adenocarcinoma DLD-1 cell lines with GI50 values of 62 ± 2 and 71 ± 2 μM, respectively [34]. Legault and Pichette [35] reported that caryophyllene alone was unable to inhibit any of the cell lines tested. However, when combined with paclitaxel (an antitumor agent used clinically to treat breast, ovarian, and lung cancers), caryophyllene could increase growth inhibition by about 91% in DLD-1 cell lines. Another study by Okada et al. [36] demonstrated that 2-methoxy-4-propylphenol induces apoptosis and is a potent inhibitor of lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) gene expression, in which, the overexpression of the COX-2 gene protects cancer cells from apoptosis.

Members of the Bcl-2 family include major cell survival and cell death regulators. Bcl-2 and Bcl-XL act as apoptosis inhibitors in the cells. While Bax functions as a proapoptotic factor and inhibits cell survival. Our data showed that PDM treatment dose-dependently decreased the expression of prosurvival proteins Bcl-2 and Bcl-xl and increased the expression of the proapoptotic molecule, Bax. The importance of Bcl-2 and Bcl-xl for protection of mitochondria during cell death process has been previously studied [37]. Excessive expression of Bax may form Mitochondrial Apoptosis-Induced Channel (MAC) and mediate the release apoptotic factor. In contrast, Bcl-2 has the ability to block apoptosis through inhibition of Bax and/or Bak. The decline in Bcl-2 expression may lead to loss of MMP which may trigger downstream caspase activation [38]. In fact, interaction of Bcl-xl with Apaf1 has a principle role in cell survival through inhibition of Apaf1-dependent caspase-9 activation [39]. Hence, upregulation of Bax and downregulation of Bcl-2 and Bcl-xl molecules by PDM treatment may lead to MMP loss, caspase cascade activation, and subsequent DNA fragmentation.

To come to a conclusion, the evidence of LDH release, MMP suppression, elevation in the level of cytochrome c, and activation of caspases demonstrated the promising anticancer activity of PDM against MCF-7 human breast cancer cell line via cell cycle arrest and apoptosis induction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Putri Narrima and Mohammadajavad Paydar have contributed equally to this work.

Acknowledgments

This research is supported by High Impact Research Grant (UM-MOHE UM.C/HIR/MOHE/SC/09 and UM.C/HIR/MOHE/EDUCATION/07) from the Ministry of Higher Education Malaysia and the University of Malaya Postgraduate Research Fund (PG093–2012B).

References

[1] M. H. Forouzanfar, K. J. Foreman, A. M. Delossantos et al., “Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis,” The Lancet, vol. 378, no. 9801, pp. 1461–1484, 2011.
[2] J. Ferlay, H.-R. Shin, F. Bray, D. Forman, C. Mathers, and D. M. Parkin, “Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008,” International Journal of Cancer, vol. 127, no. 12, pp. 2893–2917, 2010.
[3] M. Paydar, Y. L. Wong, B. A. Moharam, W. F. Wong, and C. Y. Looi, “In vitro anti-oxidant and anti-cancer activity of methanolic extract from Sanchezia speciosa leaves,” Pakistan Journal of Biological Sciences, vol. 16, no. 20, pp. 1212–1215, 2013.
[4] H. D. Soule, J. Vazquez, A. Long, S. Albert, and M. Brennan, “A human cell line from a pleural effusion derived from a breast carcinoma,” Journal of the National Cancer Institute, vol. 51, no. 5, pp. 1409–1416, 1973.
[5] C. Yang, S. Trent, V. Ionescu-Tiba et al., “Identification of cyclin D1– and estrogen-regulated genes contributing to breast carcinogenesis and progression,” Cancer Research, vol. 66, no. 24, pp. 11649–11658, 2006.
[6] K. H. Lee, “Antineoplastic agents and their analogues from Chinese traditional medicine,” in Human Medicinal Agents from Plants, A. D. Kinghorn and M. Balandrin, Eds., vol. 534 of ACS Symposium Series, pp. 170–190, American Chemical Society, Washington, DC, USA, 1993.
[7] K.-H. Lee, “Anticancer drug design based on plant-derived natural products,” Journal of Biomedical Science, vol. 6, no. 4, pp. 236–250, 1999.
[8] S. A. Abdullah and A. A. Hezri, “From forest landscape to agricultural landscape in the developing tropical country of Malaysia: pattern, process, and their significance on policy,” Environmental Management, vol. 42, no. 5, pp. 907–917, 2008.
[9] H. Ding, Y.-W. Chin, A. D. Kinghorn, and S. M. D’Ambrosio, “Chemopreventive characteristics of avocado fruit,” Seminars in Cancer Biology, vol. 17, no. 5, pp. 386–394, 2007.
[10] H. Kawagishi, Y. Fukumoto, M. Hatakeyama et al., “Liver injury suppressing compounds from avocado (Persea americana) leaf aqueous extract in mice,” Phytotherapy Research, vol. 20, no. 8, pp. 696–700, 2006.
[11] B. Nayak, S. Raju, and A. C. Rao, “Wound healing activity of Persea americana (avocado) fruit: a preclinical study on rats,” Journal of Wound Care, vol. 17, no. 3, pp. 123–125, 2008.
[12] O. Adeyemi, S. Okpo, and O. Ogunti, “Analgesic and anti-inflammatory effects of the aqueous extract of leaves of Persea americana Mill (Lauraceae),” Fitoterapia, vol. 73, no. 5, pp. 375–380, 2002.
[13] V. Bondet, W. Brand-Williams, and C. Berset, “Kinetics and mechanisms of antioxidant activity using the DPPH free radical
Evidence-Based Complementary and Alternative Medicine

C. Y. Looi, M. Imanishi, S. Takaki et al., “Octa-Arginine mediates...” Journal of Agricultural and Food Chemistry, vol. 49, no. 10, pp. 4619–4626, 2001.

M. Jacobson, E. C. Uebel, W. R. Lusby, and R. M. Waters, “Optical isomers of α-copaene derived from several plant sources,” Journal of Agricultural and Food Chemistry, vol. 35, no. 5, pp. 798–800, 1987.

F. Mtnuzi, E. D. Dikio, B. Makhwaje, A. Sipamla, and S. J. Modise, “GC-MS analysis of hexane extract of Bolusanthus speciosus stem bark,” Asian Journal of Plant Science and Research, vol. 3, no. 2, pp. 27–30, 2013.

V. R. Gummadi, S. Rajagopalan, C. Y. Looi et al., “Discovery of 7-azaindole based anaplastic lymphoma kinase (ALK) inhibitors: wild type and mutant (L196M) active compounds with unique binding mode,” Bioorganic & Medicinal Chemistry Letters, vol. 23, no. 17, pp. 4911–4918, 2013.

C. Y. Looi, M. Imanishi, S. Takaki et al., “Octa-Arginine mediates...” Journal of Agricultural and Food Chemistry, vol. 49, no. 10, pp. 4619–4626, 2001.

M. M. Rahman, M. Garvey, L. J. Piddock, and S. Gibbons, “Antibacterial terpenes from the oleo-resin of Commiphora molmol (Engl.),” Phytotherapy Research, vol. 22, no. 10, pp. 1356–1360, 2008.

Y. Zhang, M. Moerkens, S. Ramaihagari et al., “Elevated insulin-like growth factor 1 receptor signaling induces antiestrogen resistance through the MAPK/ERK and PI3K/Akt signaling routes,” Breast Cancer Research, vol. 13, no. 3, article R52, 2011.

S.-T. Chang, D. S.-Y. Wu, S.-G. Shiah, Y.-H. Kuo, and C.-J. Chang, “Cytotoxicity of extracts from Taiwania cryptomerioides heartwood,” Phytochemistry, vol. 55, no. 3, pp. 227–232, 2000.

J. Legault, W. Dahl, E. Debton, A. Pichette, and J.-C. Madelmont, “Antitumor activity of balsam fir oil: production of reactive oxygen species induced by α-humulene as possible mechanism of action,” Planta Medica, vol. 69, no. 5, pp. 402–407, 2003.

J. Legault and A. Pichette, “Potentiating effect of β-caryophyllene on anticancer activity of α-humulene, isocaryophyllene and paclitaxel,” Journal of Pharmacy and Pharmacology, vol. 59, no. 12, pp. 1643–1647, 2007.

N. Okada, A. Hirata, Y. Murakami, M. Shoji, H. Sakagami, and S. Fujisawa, “Induction of cytotoxicity and apoptosis and inhibition of cyclooxygenase-2 gene expression by eugenol-related compounds,” Anticancer Research, vol. 25, no. 5, pp. 3263–3269, 2005.

S. Shimizu, Y. Eguchi, W. Kamene, and K. Miike et al., “Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors,” Oncogene, vol. 13, no. 1, pp. 21–29, 1996.

J. Yang, X. Liu, K. Bhalla et al., “Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked,” Science, vol. 275, no. 5303, pp. 1129–1132, 1997.

Y. Hu, M. A. Benedict, D. Wu, N. Inohara, and G. Nunez, “Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation,” Proceedings of the National Academy of Sciences of the United States of America, vol. 95, no. 8, pp. 4386–4391, 1998.