

Apoptosis Activity of *Persea declinata* (Bl.) Kosterm Bark Methanolic Crude Extract

P. Narrima, C. Y. Looi, M. A. Mohd, H. M. Ali

Abstract—*Persea declinata* (Bl.) Kosterm is a member of the *Lauraceae* family, widely distributed in Southeast Asia. It is from the same genus with avocado (*Persea americana* Mill), which is widely consumed as food and for medicinal purposes. In the present study, we examined the anticancer properties of *Persea declinata* (Bl.) Kosterm bark methanolic crude extract (PDM). PDM exhibited a potent antiproliferative effect in MCF-7 human breast cancer cells, with an IC₅₀ value of 16.68 µg/mL after 48h of treatment. We observed that PDM caused cell cycle arrest and subsequent apoptosis in MCF-7 cells, as exhibited by increased population at G₀/G₁ phase, higher lactate dehydrogenase (LDH) release, and DNA fragmentation. Mechanistic studies showed that PDM caused significant elevation in ROS production, leading to perturbation of mitochondrial membrane potential, cell permeability, and activation of caspases-3/7. On the other hand, real-time PCR and Western blot analysis showed that PDM treatment increased the expression of the proapoptotic molecule, Bax, but decreased the expression of prosurvival proteins, Bcl-2 and Bcl-xL, in a dose-dependent manner. These findings imply that PDM could inhibit proliferation in MCF-7 cells via cell cycle arrest and apoptosis induction, indicating its potential as a therapeutic agent worthy of further development.

Keywords—Antiproliferative, apoptosis, MCF-7 human breast cancer, *Persea declinata*.

I. INTRODUCTION

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 [1], [2].

In many countries, medicinal plants are still collected from wild vegetation. In Malaysia, about 15% of estimated 14,500 species of flowering plants have been claimed to have medicinal properties. However, only a handful has been studied for their potential bioactivities and is currently cultivated by various farming communities, but there are still many more plants to be discovered. One of the undiscovered potentially bioactive plants is the *Persea declinata* (Bl.) Kosterm (common names: medang inai, medang tanah, kayu helah, huru manok, huru leu-ur, and meang telut), a member

of the *Lauraceae* family, and is widely distributed in Borneo, Java, Malaysia (Penang, Kelantan, Terengganu, Pahang, Selangor) and Singapore. 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 is known to possess various potential cancer preventive phytochemicals [3]. While countless studies have been done on avocado for anticancer properties, *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.

II. MATERIALS AND METHODS

A. 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).

B. 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.

C. 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 obtained 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

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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.

D. Extraction

50 grams of grounded bark of *Persea declinata* (Bl.) Kosterm was extracted with 500mL 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 15mL volume using a rotary evaporator and further frozen and freeze-dried for another 48 hours.

E. MTT Cell Viability Assay

The cytotoxic effect of PDM was assessed by MTT cell viability assay against different cancer cells [4]. 1.0×10^4 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 2mg/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 [5]. 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.

F. Real-Time Cell Proliferation

In vitro proliferation of PDM-treated and untreated cells was surveyed using a Real-Time Cellular Analysis (JuLi Br) system. 2.0×10^5 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.

G. 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 30min, 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.

H. Cell Cycle Analysis

1×10^4 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. 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.

I. Reactive Oxygen Species (ROS) Assays

1×10^4 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 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.

J. Nuclear Morphology, Membrane Permeability, and Mitochondrial Membrane Potential (MMP) Assays

Cellomics multiparameter cytotoxicity 3 kit (Thermo Scientific) was used as described previously [6]. 1×10^4 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 Cellomics ArrayScan HCS reader (Thermo Scientific).

K. DNA Fragmentation Analysis by Acridine Orange

1×10^4 cells per well were plated on 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 15min. The cells were then fixed and visualized and images were captured using a camera connected to a fluorescent microscope.

L. Bioluminescent Assays for Caspase-3/7 Activities

Dosedependent 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 1×10^4 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 μ L 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, Mannedorf, Switzerland) microplate reader.

M. Western Blot Analysis

SDS-PAGE and Western blot analyses were done as described with slight modifications [7]. Briefly, 24 hours posttreatment, cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with freshly added 10mM β -glycerophosphate, 1mM sodium orthovanadate, 10mMNaF, and 1mMphenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (Santa Cruz, CA) and loaded onto 10% polyacrylamide gel. Proteins were then transferred to microporous polyvinylidene difluoride (PVDF) membrane (Milipore). Membranes were incubated in 5% BSA (Sigma) blocking buffer for 1 h at room temperature. Incubations with primary antibody were carried out overnight at 4°C. Immunoblotting was 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 (10min 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.

N. Statistical Analysis

Experimental values were presented as the means \pm 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 $P < 0.05$.

III. RESULTS AND DISCUSSION

A. 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 I shows the IC₅₀ values after 48 hours of treatment with PDM. Highest cytotoxicity was observed in MCF-7 breast cancer cells (IC₅₀ = 16.68 \pm 0.89). 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 [8], [9]. 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 (Fig. 1).

TABLE I
EFFECT OF *PERSEA DECLINATA* (BL.) KOSTERM BARK CRUDE EXTRACT ON CELLS EXPRESSED AS IC₅₀ VALUES IN 48 HOURS MTT ASSAY

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

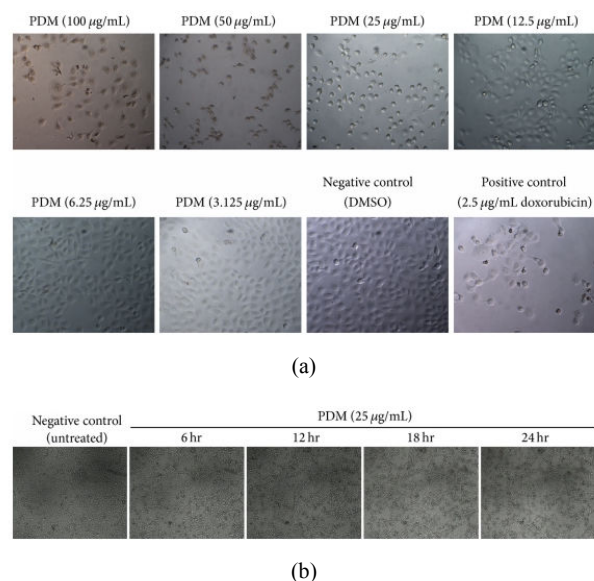


Fig. 1 PDM induced significant reduction of cell number, cell shrinkage, and apoptotic body formation in treated MCF-7 cells. (a) MCF-7 cells were treated with DMSO, doxorubicin, or different concentrations of PDM for 24h. Live cell images indicating cell shrinkage and apoptosis in a dose-dependent manner. (b) MCF-7 cells were treated with DMSO or PDM (25 μ g/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

B. 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 24h. PDM induced a significant elevation in LDH release, demonstrating cytotoxicity at 25 and 50 μ g/mL concentrations compared to the control cells (Fig. 2).

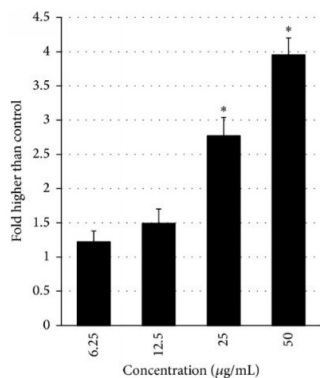


Fig. 2 The LDH release assay revealed the significant cytotoxicity of PDM on MCF-7 cells at 25 µg/mL and 50 µg/mL concentrations

C. Effect of PDM on Cell Cycle

We investigated the cell cycle arrest of MCF-7 cells treated with different concentrations of PDM. The histogram plot on Hoechst total intensity 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 (Fig. 3). These results show that PDM induced G0/G1 arrest in MCF-7 after 24 hours.

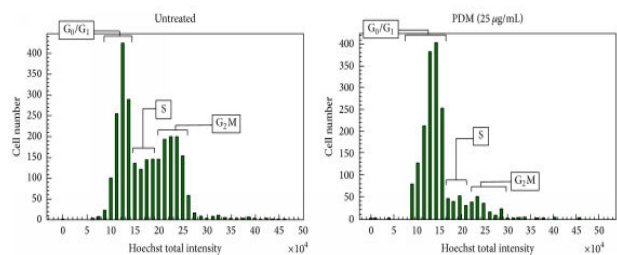


Fig. 3 Cells were stained with Hoechst and the distribution in G0/G1, S, and G2/M cell cycle phases was determined with Cellomics High Content Screening

D. 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 Fig. 4, the levels of DHE increased significantly in MCF-7 cells treated with PDM, indicating higher ROS production.

From these results, 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.

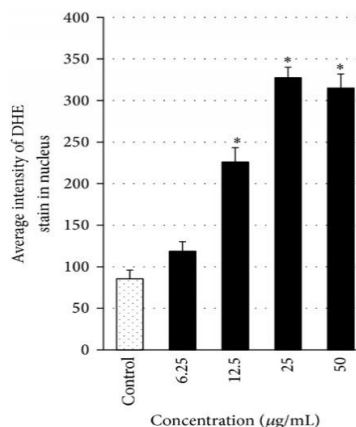
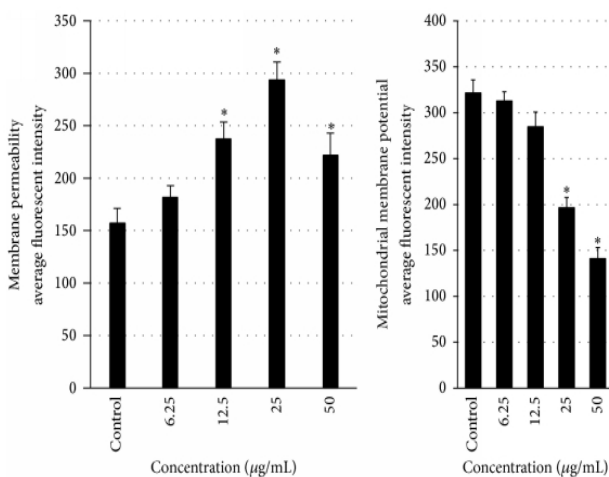


Fig. 4 Representative bar charts indicating dose-dependent increased ROS in the nucleus of the PDM-treated MCF-7 cells

E. PDM Decreased Mitochondrial Membrane Potential (MMP) and Increased Cell Membrane Permeability

As the main source of cellular ROS and adenosine triphosphate (ATP), mitochondria are the key regulators of mechanisms controlling the survival or death of cells. We used mitochondrial membrane potential (MMP) fluorescent probes to examine the function of mitochondria in treated and untreated MCF-7 cells. As shown in Fig. 5, the dose-dependent reduction of MMP fluorescence intensity reflects that the MMP is gradually destroyed in response to higher PDM concentration. On the other hand, a significant increase in cell membrane permeability was also observed in PDM treated cells after 24 hours of treatment (Fig. 5).

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, as previously experimented.



(a)

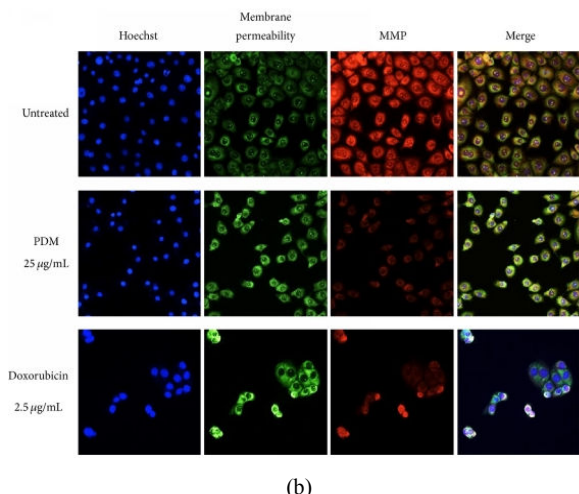
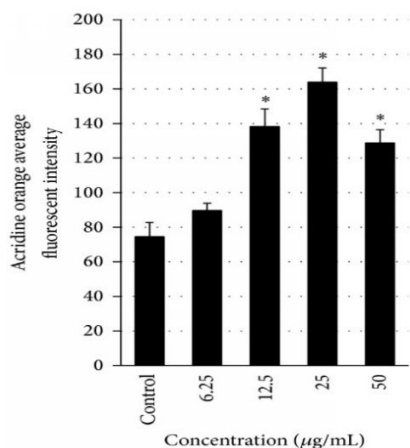


Fig. 5 (a) Representative bar charts indicating dose-dependent increased cell permeability and reduced MMP in the PDM-treated MCF-7 cells. (b) Representative images of MCF-7 cells treated with DMEM, doxorubicin, or PDM for 24 h and stained with Hoechst 33342 for nuclear, membrane permeability, and mitochondrial membrane potential (MMP) dyes. PDM induced a noteworthy elevation in membrane permeability and a marked reduction in MMP (magnification: 200x)

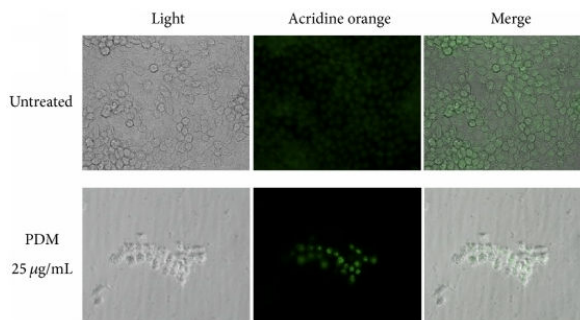
F. PDM Induced DNA Fragmentation

Acridine orange (AO) is used to determine DNA integrity. We stained MCF-7 16 cells with acridine orange dye after 24 hours of treatment. Figs. 6 (a) and (b) showed a dose-dependent increase of nucleus restricted acridine orange dye in PDM-treated cells, indicating 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. Caspase 3/7 normally cleaves several target proteins such as PARP (the enzyme responsible for repairing DNA) and leads to apoptotic DNA fragmentation.



(a)



(b)

Fig. 6 (a) 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. (b) Acridine orange DNA fragmentation analysis. The cells were treated with DMEM or PDM for 24 h and stained with acridine orange dye

G. 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 Fig. 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. 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 [10].

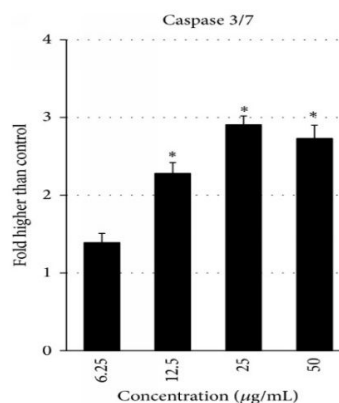


Fig. 7 Relative luminescence expression of caspases 3/7 in MCF-7 cells treated with various concentrations of PDM

H. PDM Modulated Expression of Bcl-2, Bcl-xl, and Bax

Bcl-2 includes a family of proteins that regulate Mitochondrial Outer Membrane Permeabilization (MOMP). They include antiapoptotic molecules such as Bcl-2 and Bcl-xl, which could preserve cell survival and proapoptotic molecules like Bax that inhibit cell survival. To examine if PDM initiated apoptosis by affecting the cellular level of these

molecules, Western blot analysis was performed using untreated or extract-treated breast cancer cells. Our data showed that PDM dose-dependently upregulated Bax and downregulated the expression level of Bcl-2 and Bcl-xl in MCF-7 cells (Fig. 8).

The importance of Bcl-2 and Bcl-xl for protection of mitochondria during cell death process has been previously studied [11]. 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 [12]. In fact, interaction of Bcl-xL with Apaf1 has a principle role in cell survival through inhibition of Apaf1-dependent caspase-9 activation [13]. 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.

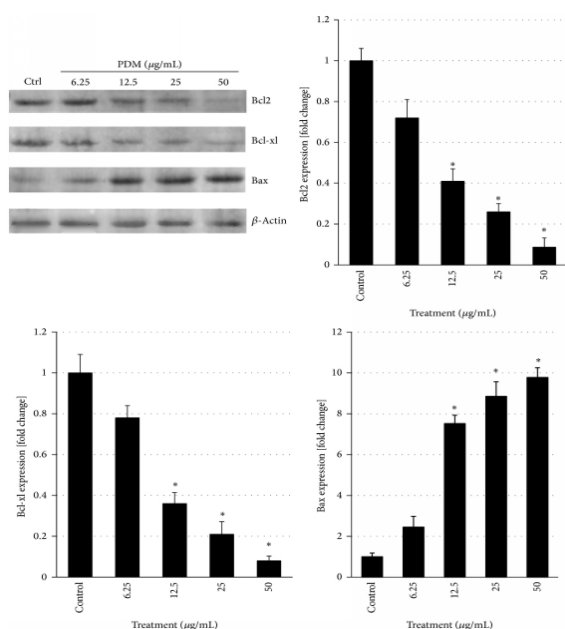


Fig. 8 Induction of apoptosis by reducing the expression level of prosurvival molecules (Bcl-2, Bcl-xl) and increasing the expression of proapoptotic molecule (Bax). MCF-7 cells were treated with DMSO or different concentrations of PDM for 24 h. Western blot results showing the expression levels of Bcl-2, Bcl-xl, and Bax in PDM or DMSO-treated MCF-7 cells. β -Actin serves as a loading control

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.

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