Polyalthia longifolia Methanolic Leaf Extracts (PLME) induce apoptosis, cell cycle arrest and mitochondrial potential depolarization by possibly modulating the redox status in HeLa cells

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ABSTRACT

Medicinal plants have been accepted as a gold mine, with respect to the diversity of their phytochemicals. Many medicinal plants extracts are potential anticancer agents. Polyalthia longifolia var. angustifolia Thw. (Annonaceae) is one of the most significant native medicinal plants and is found throughout Malaysia. Hence, the present study was intended to assess the anticancer properties of P. longifolia leaf methanolic extract (PLME) and its underlying mechanisms. The Annexin V/PI flow cytometry analysis showed that PLME induces apoptosis in HeLa cells in dose-dependent manner whereas the PI flow cytometric analysis for cell cycle demonstrated the accumulation of cells at sub G0/G1, G0/G1 and G2/M phases. Investigation with JC-1 flow cytometry analysis indicated increase in mitochondria membrane potential depolarisation corresponding to increase in PLME concentrations. PLME was also shown to influence intracellular reactive oxygen species (ROS) by exerting anti-oxidant (half IC50) and pro-oxidant (IC50 and double IC50) effect against HeLa cells. PLME treatment also displayed DNA damage in HeLa cells in concentration depended fashion. The proteomic profiling array exposed the expression of pro-apoptotic and anti-apoptotic proteins upon PLME treatment at IC50 concentration in HeLa cells. Pro-apoptotic proteins; BAX, BAD, cytochrome c, caspase-3, p21, p27 and p53 were found to be significantly up-regulated while anti-apoptotic proteins; BCL-2 and BCL-w were found to be significantly down-regulated. This investigation postulated the role of p53 into mediating apoptosis, cell cycle arrest and mitochondrial potential depolarisation by modulating the redox status of HeLa cells.

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1. Introduction

Medicinal plants rich with various phytochemicals have received great attention in the development of anticancer agents due to their numerous medicinal properties [1]. It is presently estimated that >50% of all patients diagnosed with cancer explore complementary and alternative medicine, especially herbal medicine [2] Although many medicinal plant’s curative properties were reported in the literature but there are still various medicinal plants need comprehensive scientific study. Accordingly, certifying the traditional use of these medicinal plants and studying the possible mechanism of bioactivities can offer a rich source of new pharmaceutical products particularly in the development of anticancer agents. Hence, the present study was intended to assess the Polyalthia longifolia var. angustifolia Thw. (Annonaceae) leaf methanolic extract (PLME) anticancer properties and the underlying mechanisms. P. longifolia is one of the most significant native medicinal plants and is found throughout Malaysia. Various phytochemicals such as diterpenes, alkaloids, steroids, and miscellaneous lactones have been isolated from its bark. The stem bark extracts and isolated compounds have been studied for various biological activities, such as cytotoxicity, and antibacterial and antifungal activity [3,4]. Various bioactivities of this important medicinal plant was recently reported in the literature such as antioxidant activity, hepatoprotective activity [5] genoprotective

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activity, acute oral toxicity [6] and in vivo radioprotective activity [4] of leaf extract. Furthermore, P. longifolia is widely used in traditional medicine as a febrifuge and tonic [7]. Our preliminary study showed that the standardized methanolic leaf extract of P. longifolia (PLME) has exhibited cytotoxicity against HeLa cell line in vitro [8]. However, possible pathway and detailed mechanisms of action of P. longifolia leaves have not been investigated this far. Therefore this study was conducted to establish the possible apoptotic pathways induced in HeLa cells by P. longifolia leaves.

The increase search for competent anticancer agents now resides greatly upon the strategic to identify agents with selective ability in cancer cell annihilation. Cancer is an outcome to a loss of balance between cell proliferation and cell death during which a normal cell becomes malignant by evading cell death or apoptosis [9]. Due to the fact that apoptotic mechanism can be altered to influence activation of cell death in cancers, such strategic becomes feasible with explorations continuing to confine the cytototoxicity effect to only cancer cells and minimize the tendency of side effects in normal cells. Therefore, the important mechanisms in the avoidance and suppression of cancer are inhibition of cell cycle machinery and induction of apoptosis in the abnormal cells [1]. The induction of apoptosis is highly preferred pathway for therapeutic intervention even with the emergence of alternative paths leading to cell death due to its limited side effects to the adjacent normal cells [10]. Moreover, cell cycle arrest by therapeutic agents in the Sub G1, G0 and G1 phases can lead to apoptosis [11]. In this study, HeLa cells were exposed to varying PLME concentrations to investigate whether the growth inhibitory effects of the PLME are due to changes in cell cycle progression and/or apoptosis with their underlying molecular pathways involved in the regulation of cell death. Understanding of the underlying molecular pathways of cell cycle machinery and induction of apoptosis in cancer cells could provide new rational strategy and targets for effective cancer therapy.

2. Material and methods

2.1. Plant material collection and extraction

The leaves of P. longifolia were collected from various areas in Universiti Sains Malaysia and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a sample was deposited (Voucher specimen: USM/HERBARIUM/11306). The leaves were cut, washed with distilled water and oven-dry at 30 °C for 7 days. The dried leaves are then ground into fine powder. A sample of 100 g of plant powder was soaked in 400 mL of methanol at RT (23 °C ± 2) for 7 days. The filtrate from each extraction was concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at 40 °C and the concentrated extract was finally poured into Petri dishes and brought to dryness at 40 °C in oven. The resultant extract paste is stored at room temperature (RT) in dark.

2.2. Chemical analysis of PLME using abbreviation for gas chromatography mass spectrometry (GC–MS) analysis

The GC–MS profiling was performed using a 6890N Network GC System (Agilent Technologies, USA) equipped with a methyl phenyl polysiloxane cross-linked 5% phenyl methyl silicone (30 m long, 0.25 mm internal diameter, 0.25 μm film thickness) capillary column. The mass spectrometer attached to the GC model was a 5973I Network Mass selective Detector (Agilent Technologies, USA). The detector and injector temperatures were both maintained at 280 °C. The quadrupole mass spectrometer scanned over the range of 40–700 m/z with the ionizing voltage of 70 eV and ionization current of 150 mA using an ion source temperature of 200 °C. The GC conditions, which were optimized on standard mixtures of pure compounds, were as follow: initial temperature 70 °C, 2 min isothermal, ramp at 20 °C min–1 up to 280 °C for 20 min. The carrier gas was helium at a flow rate of 1 mL/min. The GC–MS system was computer-controlled by the Enhanced ChemStation software (Agilent Technologies, USA) which utilises The National Institute of Standard and Technology (NIST) and Wiley search libraries.

2.3. Cell culture and treatment

HeLa cell line was obtained from American Tissue Culture Collection (ATCC, USA) and was maintained in Dulbecco Modified Eagle Medium (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS), glutamine (2 mM), penicillin (100 units/mL) and streptomycin (100 μg/mL). The cells were cultured at 37 °C in a humidified 5% CO2 incubator. Ensuing from MTT and CyQUANT cytotoxicity assays, the IC50 concentration of PLME was determined for HeLa cells. Cells were then treated with PLME at the concentration of half IC50 (11.00 μg/mL), IC50 (22.00 μg/mL) and double IC50 (44.00 μg/mL) [8]. PLME samples were solubilized in sterile filtered Dimethyl sulfoxide (DMSO) (0.2% in the culture medium) prior to addition to the culture media.

2.4. Flow cytometry analysis of apoptosis/necrosis by using annexin V-FITC/propidium iodide (PI) staining method

Apoptotic cells are able to be distinguished based on their everted location of inner phosphatidylserine found on cell surface with the aid of Annexin V-FITC (fluorescein isothiocyanate) antibody. Briefly, HeLa cells were seeded into 25 cm2 cell culture flasks at a density of 1 × 106 cells before allowed to attach overnight and treated with 1/2 × IC50, IC50 and 2 × IC50 of PMEAF for 24 h. Parallel cells cultured without treatment but suspended in the same volume of 0.2% DMSO were prepared and regarded as vehicle control (negative controls). Measurement of live, apoptotic and necrotic cells was performed by using Annexin V and PI staining (BD Pharmingen, San Diego, USA), in accordance with the manufacturer’s instructions. Following incubation, cells were harvested by adding trypsin and washed twice with PBS. The cell pellet was resuspended in 1 mL of 1 × Annexin V binding buffer and subsequently incubated with 5 μL of Annexin V conjugated FITC and 5 μL of PI staining solution for 15 min at room temperature (23 ± 2 °C) in dark. Next, cells were analysed by flow cytometry within 1 h. Flow cytometry was performed on FACS Cantoll (BD BioSciences, San Jose, USA). Fifty thousand events were collected per sample and data were analysed using FACSDiva Version 6.1.3 software (BD BioSciences, San Jose, USA).

2.5. Cell cycle analysis by flow cytometry

Propidium Iodide Flow Cytometry method was used to determine the effect of PLME on HeLa cell cycle phases [12]. Briefly, HeLa cells were seeded into 25 cm2 cell culture flasks at a density of 1 × 106 cells before allowed to attach overnight and treated with 1/2 × IC50, IC50 and 2 × IC50 of PMEAF for 24 h. Parallel cells cultured without treatment but suspended in the same volume of 0.2% DMSO were prepared and regarded as vehicle control (negative controls). Harvested cells were washed with PBS and stained with PI using a CycleTEST Plus DNA reagent kit (BD BioSciences, San Jose, USA) according to manufacturer’s instructions. The linearity FACS Cantoll (BD BioSciences, San Jose, USA) was tested using BD DNA QC Particles kit (BD BioSciences, San Jose, USA). Doublets, disintegrated nuclei and other cell debris were removed from analysis by gating forward and side scatter profile of samples. The gates were uniformly maintained across all samples.
in each run. For each sample, fifty thousand events (nuclei) were collected and the resulting histograms with percentages of cells in G0/G1, S and G2/M phases were analyzed using Modfit software Version 3.2 (BD BioSciences, San Jose, USA).

2.6. DCF assay for reactive oxygen species (ROS) determination

The 2', 7'-dichlorodihydrofluorescein (DCF) assay was used to determine the effect of PLME on the generations of intracellular ROS in HeLa cell [13,14]. The generations of intracellular oxygen species ROS in HeLa cells after treatment with PLME was estimated using Intracellular ROS Assay Kit (Oxisellect Cell Biologs Inc., San Diego, USA), that engages the cell-permeable fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to penetrate cell membrane and be deacetylated upon the presence of intracellular esterases to nonfluorescent 2',7'-dichlorodihydrofluorescein (DCFH). DCFH is promptly oxidized by ROS occurrence into greater fluorescent DCF, which is easily detectable. The fluorescence strength is directly proportional to the ROS level within cell cytoplasm. Briefly, HeLa cells were seeded into 96-well plates at a density of 5 x 10^3 cells/well before allowed to attach overnight and 100 μL of 1 x DCFH-DA solution was added into each well and further incubated for 1 h in dark. Following DCFH-DA exposure, cells were then washed with PBS and treated with 100 μL of half IC50, IC50 and double IC50 concentrations of PLME for 24 h. Parallel sets of cells without treatment but suspended in the same volume of 0.2% DMSO were prepared and regarded as vehicle control (negative controls). The fluorescence was observed at regular intervals at an excitation wavelength of 480 nm and an emission wavelength of 530 nm in a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, USA). The quantity of DCF formed was calculated from a calibrated curve constructed using a legitimate DCF standard. The relative DCF intensity was quantified as the percentage ratio of the fluorescence intensities in the wells to that in the vehicle control wells. Percentages of ROS level were expressed in terms of percentages of differences from vehicle control after background subtraction with the following equation:

\[
\text{Percentage of differences from vehicle control (％)} = \frac{\text{Treated} - \text{Vehicle control (DCF, nm)}}{\text{Vehicle control (DCF, nM)}} \times 100
\]

2.7. Comet assay for potential DNA damage

The damages to cellular DNA in individual HeLa cells that contain the fragments and strand breaks are further evaluated by using the OxiSelectTM Comet Assay Kit (Oxisellect Cell Biologs Inc., San Diego, USA). Briefly, HeLa cells were seeded into 6-well plates at a density of 1.0 x 10^5 cells/well before allowed to attach overnight. The cells were then treated with different concentrations of PLME (half IC50, IC50 and double IC50) and vehicle control (only 0.2% DMSO) which were further incubated for another 24 h. Positive control was prepared by treating HeLa cells with 10 μM of H2O2 for 15 mins. The cells were treated with trypsin and later washed with cold PBS, collected into 15 mL Falcon tubes and centrifuged at 400 x g. Prior to assay, Oxisellect Comet Agarose was melted at 90°C for 20 min and then cooled at 37°C for 20 min. The cell pellets were then combined with the Oxiselect Comet Agarose at 1:10 ratio (w/v), mixed and 75 μL of the mixture was immediately pipetted onto the Comet assay slide. The slides were dried at 4°C in the dark for 15 min followed by incubation in pre-chilled lysis solution at 4°C in the dark for another 15 min. The samples were then washed with TBE buffer [50 mM Tris, 50 mM boric acid, and 0.2 mM EDTA] and were subjected to electrophoresis at 31 V (0.96 V/cm at 300 mA) for 20 min in TBE buffer. Vista green DNA dye was used to stain the cells and the signal was detected with a BX53 fluorescent microscope (Olympus, Tokyo, Japan). Approximately 50 micrographs of cells for respective conditions were captured. The assessment of comets was completed using CASP version 1.2.2 (Zbigniew Kozaj, Poland).

2.8. Measurement of mitochondrial membrane potential (ΔΨm) by using JC-1 mito screen assay

Briefly, HeLa cells were seeded into 25 cm² cell culture flasks at a density of 1 x 10^5 cells before allowed to attach overnight and treated with half IC50, IC50 and double IC50 concentrations of PLME and then further incubated for another 24 h. Parallel culture flasks of cells without treatment but suspended in the same volume of 0.2% DMSO were prepared and regarded as vehicle control (negative controls) while positive control was prepared with cells suspended in 20 μM of CCCP (Sigma Aldrich, St. Louis, USA) for 24 h of incubation. The ΔΨm was assessed using JC-1 from BD™ MitoScreen kit (BD Biosciences, San Jose, USA) according to manufacturer’s instructions. JC-1 is a cationic dye that easily penetrates and accumulates in mitochondrial polarized membranes, serving as a sensitive way to measure ΔΨm. Cells were harvested and transferred into 15 mL Falcon tubes. The JC-1 dye was added to the cells (500 μL) in each tube and incubated at 37°C with 5% CO2 for 15 min. Following incubation, 2 mL of JC-1 Assay Buffer (BD Biosciences, San Jose, USA) was added and centrifuged at 400 x g for 5 min before resuspending in 500 μL JC-1 Assay Buffer. The flow cytometry analysis was performed on BD FACS Cantoll (BD Biosciences, San Jose, USA). Fifty thousand events were collected per sample and data were analysed using FACS Diva version 6.1.3 software (BD Biosciences, San Jose, USA). A scatter plot of red (intact ΔΨm) versus green fluorescence (decreased ΔΨm) was produced.

2.9. Human apoptotic proteins array

Participation of apoptotic proteins during apoptosis induced by PLME in HeLa cells was determined through apoptosis array using RayBio® Human Apoptosis Array G-series (RayBiotech, Inc.) Human Apoptosis Antibody Array kit (RayBiotech Inc., Norcross, USA) Following treatments with PLME at IC50 for 24 h, HeLa cells from wells (10 x 10^6 cells/treatment) were solubilized in 500 μL lysis buffer containing protease inhibitors for 30 min and sonication on ice was performed with a Bioblock Scientific Instrument (Vibra Cell 75044; 12 cycles, 20 s ON and 60 s PAUSE). Cells were then pelleted at 14,000 x g for 30 min at 4°C, and supernatants were aspirated. Protein concentration in supernatants was determined using the bicinchoninic acid assay (BCA) method (Thermo Scientific, Pierce). Protein load per membrane was approximately 600 μg/ml total protein diluted 10-fold with blocking buffer. Membranes were blocked with blocking buffer for 30 min at room temperature (23 ± 2°C). Diluted sample was applied to membranes and incubated overnight with gentle rocking at room temperature (23 ± 2°C). Membranes were washed, and a biotin-conjugated secondary antibody was added. Horseradish peroxidases (HRP) conjugated streptavidin was added for detection through enhanced chemiluminescence. Blots were imaged using ChemiDoc XR® gel imager (BioRad). Relative densities of antibodies were determined by comparing to protein loading control antibodies.

2.10. Statistical analysis

All data are expressed as mean value ± standard deviation (SD), Statistical analysis was performed using one-way ANOVA followed
by Tukey's post hoc multiple comparison tests (GraphPad Prism version 7 for Windows, GraphPad Software Inc., San Diego, USA). A $p < 0.05$ is considered statistically significant.

3. Results

3.1. Gas chromatography profile of PLME using GC–MS analysis

The PLME extract was characterized by GC–MS. The chromatographic analysis revealed 14 major peaks with four major compound namely Phenol, 2,4-bis(1,1-dimethylethyl)-; Pentadecanoic acid, 14-methyl-, methyl ester; 9-Octadecenoic acid, methyl ester, (E)- and Octadecanoic acid, methyl ester (Fig. 1, Table 1).

3.2. PLME induced apoptosis in HeLa cells

The percentage of apoptotic cells in PLME treated HeLa cells were monitored using FITC-Annexin V and propium iodide (PI) double staining by FACS (Fig. 2). The vehicle control cells visibly indicate the presence of viable cells at the highest quantity. Annexin V-FITC and PI double staining of HeLa cells treated with various IC$_{50}$ concentrations of PLME showed dose dependent increase in the number of apoptotic cells. A small degree of necrotic cells (below 1.0%) was observed throughout the treatment with various PLME concentrations. The accumulation of cells from lower left quadrant to the right panel quadrants were indicating the shift in viable cells (Annexin V-) into early and late apoptosis (Annexin V+) Fig. 2A.

Fig. 2B represents the quantitative analysis of percentages of apoptotic, necrotic and live cells which were deduced from Fig. 2A quadrants. Vehicle control cells showed the presence of 94.90 ± 0.36% viable cells, 3.10 ± 0.35% early apoptosis, 1.87 ± 0.23% late apoptosis and 0.133 ± 0.06% necrotic HeLa cells. The half IC$_{50}$ PLME treated cells showed the presence of 7.13 ± 0.415 and 15.93 ± 0.41% of total early and late apoptosis cells respectively where higher percentage was account for late apoptosis. The number of viable cells was 76.80 ± 0.61% with...
significant reduction ($p < 0.05$) compared to vehicle control cells while necrotic cells were present at the percentage of $0.17 \pm 0.10\%$. The IC$50$ concentration of PLME treatment displayed the presence of approximately 50.0% of apoptotic cells with $49.37 \pm 1.66\%$ being viable and $0.67 \pm 0.57\%$ necrotic HeLa cells. With an increase of PLME concentration, the apoptotic cells seemed to be increased by 2 folds ($26.94\%$) as compared to the apoptosis cells generated with half IC$50$ treatment. The highest apoptotic cells were produced by PLME at double IC$50$ treatment. Nearly 59.27% of apoptosis cells were generated along with $39.83 \pm 1.91\%$ of viable and $0.73 \pm 0.35\%$ of necrotic cells. Even though these apoptotic cells had increased around 9.27% compared to apoptotic cells of IC$50$, however an increment of 1.5 fold was observed in comparison to half IC$50$. Apart from that, viable cells were seen to be reduced tremendously with a percentage of 55.1% compared to viable cells found in vehicle control HeLa cells. The data from flow cytometry analysis indicates that the mode of induced cell death by PLME in HeLa was primarily apoptosis.

3.3. The effect of PLME treatment on HeLa cell cycle distribution

The flow cytometric analysis of Propidium Iodide (PI)-stained was used to investigate the effect of PLME with different concentrations (half IC$50$, IC$50$ and double IC$50$) on cell cycle progression on HeLa cells (Fig. 3A). Vehicle control cells showed normal DNA content and cell cycle distribution with values of $0.12 \pm 0.07\%$ in sub G0/G1, $50.80 \pm 0.39\%$ in G0/G1, $49.04 \pm 0.30\%$ in S and $0.16 \pm 0.09\%$ in G2/M phases (Fig. 3B). When HeLa cells were treated with half IC$50$, a sudden hike was observed at sub G0/G1, G0/G1 and G2/M peaks while a small percentage of reduction was noticed for S phase. It could be deduced that PLME is arresting cells at both G0/G1 and G2/M phases with a small increase in apoptosis (sub G0/G1). The half IC$50$ PLME treatment also significantly increases the DNA content in sub G0/G1, G0/G1 and G2/M phases. Moreover, the DNA content in S phase was exhibited to reduce in contrast to half IC$50$ treatment. The IC$50$ concentration treatment of PLME significantly increases cell accumulation in sub G0/G1, G0/G1 and G2/M phases while the S phase displayed a reduction in vehicle control (S phase) and a small increment of 1.28% in comparison to half IC$50$ treatment group. The histogram of cells treated with double IC$50$ concentration of PLME was significantly increase in sub G0/G1, G0/G1 and G2/M peaks. At this point, G0/G1 peak indicates the highest percentage of cell arrest, followed by G2/M peak. Sub G0/G1 peak has increased as well indicating the presence to apoptosis. The cell accumulation in sub G0/G1 was increased at the values of 17.79 \pm 0.60\%. These results suggest that PLME arrests HeLa cell cycle at G0/G1 and G2/M phases in concentration dependent manner at the same time increases the accumulation at sub G0/G1 phase.

3.4. PLME affects mitochondrial membrane potential $\Delta \psi_m$ in HeLa cells

The effect of PLME on $\Delta \psi_m$ in HeLa cells was also studied to define whether PLME induced-ROS production corresponds with loss of $\Delta \psi_m$, which is considered as an early intracellular happening during beginning of apoptosis [15]. The changes in $\Delta \psi_m$, induced by PLME were determined using the fluorescent probe, JC-1. The vehicle control cells with JC-1 found in Fig. 4A predominantly exhibit red fluorescence and were found to be composed of $96.40 \pm 0.66\%$ (P2) and $1.80 \pm 0.22\%$ (P3). Treatment with PLME at half IC$50$, IC$50$ and double IC$50$ for 24 h, displayed a tremendous shift from decreased red fluorescent (P2) to increased green fluorescent (P3). At half IC$50$ treatment, the cells at P2 population decrease to $11.43 \pm 1.26\%$ while the P3 population increased to $86.43 \pm 0.87\%$ (Fig. 4B). In similar manner, the population of P2 of treated PLME at IC$50$ concentration also observed a decreased to $2.50 \pm 0.70\%$ with P3 population increasing to $94.23 \pm 0.55\%$. At double IC$50$ concentration, the treated group displayed extreme reduction in P2 population by achieving $0.40 \pm 0.10$ whereas; the P3 population observes an increase to $98.27 \pm 1.12\%$. At the highest double IC$50$ concentration, almost all the red fluorescence has shifted into green fluorescence. The change from red to green fluorescence indicates a decrease in $\Delta \psi_m$. The CCCP, an uncoupling agent was utilised for this study as positive control. The CCCP treated group generated non-apoptotic red fluorescent (P2) with the percentage of $0.97 \pm 0.21\%$ and apoptotic green fluorescent (P3) with the percentage of $98.53 \pm 0.76\%$. The effects of CCCP on $\Delta \psi_m$ seemed to be similar to the PLME double IC$50$ concentration treatment without any significant differences ($p > 0.05$). This proves that at highest concentration, PLME is capable of exerting effects to $\Delta \psi_m$ analogue to CCCP. The findings here proposed that PLME induces apoptosis in HeLa cells through the involvement of mitochondrial signal transduction pathway.

3.5. ROS generation in hela cells treated with various concentration of PLME

ROS pays a critical role in mediating cytotoxicity induced by natural chemotherapeutic agents and could contribute on cell cycle arrest or cellular apoptosis. Hence, the effect of PLME treatment on ROS generation in HeLa cells were investigated (Fig. 5). At the lowest concentration of half IC$50$, the PLME appeared to reduce the generation of intracellular ROS to $-35.53 \pm 3.31\%$. 

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**Table 1**

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Interestingly, when the concentration of PLME was increased to IC$_{50}$, the intracellular ROS generation in HeLa was increased to 42.07 ± 8.71%. In addition, HeLa cells treated with double IC$_{50}$ of PLME was able to increase the generation of intracellular ROS to 177.64 ± 15.10%. Meanwhile, the positive control H$_2$O$_2$ displays highest production of ROS with an increase of 231.27 ± 8.47% compared to vehicle control HeLa cells. The findings here indicated that PLME at the lowest concentration displayed anti-oxidant activity by scavenging ROS and contrarily at high concentrations exhibited tremendous pro-oxidant activity via increase of intracellular ROS in HeLa cells. This ability proves that PLME is capable of modulating the redox state of HeLa cells.

3.6. PLME induces DNA fragmentation in HeLa cells

DNA fragmentation is one of the most important apoptosis hallmarks which can be detected by comet assay as illustrated in Fig. 6A. The vehicle control HeLa cells did not indicate any DNA damage and therefore the nucleus regions stained with fluorescent dye appeared in spherical forms (Fig. 6A). However, treatment with half IC$_{50}$ of PLME begins to initiate comet tail formation in HeLa cells. The number of cells with comet tails seems to increase parallel to the increasing concentration of IC$_{50}$ and double IC$_{50}$. Moreover, majority of cells treated with double IC$_{50}$ of PLME appeared with comet tails. In addition, various DNA fragmentation
parameters measured includes number of comet formations, comet tail length, comet tail moment, percentage of head and tail DNA and are given in Fig. 6B–D. As expected, the DNA fragmentation parameters such number of comet formations, comet tail length, comet tail moment, percentage of head and tail DNA were increased in concentration dependent manner upon treatment with half IC50, IC50 and double IC50 of PLME (Fig. 6B–D). This findings indicating that the DNA fragmentation seems to increase parallel to the increasing concentration of PLME. Conclusively, the number of cells with comet tail over 100 single cells tends to increase with increasing concentrations of PLME. As predicted, HeLa cells treated with H2O2, a known DNA damage-inducing agent that served as positive control for this investigation, also exhibited large number of cells with comet tails.

3.7. Effects of PLME on apoptosis-related proteins expression in HeLa cells

The expression of various pro- and anti-apoptotic proteins in HeLa cells was investigated in PLME (22.00 μg/mL) treated and vehicle control cells by Raybio Array. Fig. 7A depicts the representative image of apoptotic protein expressions from vehicle controls.

Fig. 3. PLME affects cell cycle distribution in dose dependent manner. A Flow cytometry analysis showed various degrees of changes in their cell cycle phases. Each histogram includes the quantified percentages of DNA content and cell cycle distribution for sub G0/G1, G0/G1, S, and G2/M phases. Images are the representative of three independent experiments. B Quantitative analysis of cell cycle distribution indicating untreated and PLME treated cells. Different alphabets (a-m) indicate significant differences (P < 0.05) using one-way ANOVA followed by Turkey’s multiple comparison tests.
control and PLME-treated HeLa cell lysates whereas Fig. 7B illustrates the quantitative analysis pertaining to apoptotic proteins change fold (treated/vehicle control). The threshold values for fold-change were set at ≤0.667 (up-regulation) or ≥1.5 (down-regulation) [16]. Based on the protein profile analysis, 9 proteins namely BAX, BAD, BCL-2, BCL-w, caspase-3, cytochrome c, p21, p27 and p53 were found to be involved in apoptosis. Of these proteins, 7 consisting of BAD, BAX, caspase-3, cytochrome c, p27 p21 and p53 were overexpressed upon treatment with PLME. On contrary, the remaining 2 proteins (BCL-2 and BCL-w) were found to be down-regulated significantly (p < 0.05). The highest fold change of 3.700 was observed for p53 protein while the lowest fold-changes of 0.131 were observed for BCL-w protein.

4. Discussion

Apoptosis is tracked continuously as the most scrutinised mechanism in the biological field ever since it was coined by Kerr and colleagues in 1970s [17]. The process is comprehensively studied as such that it was found role-playing in both physiological and pathological state [18,19]. Every now and then, millions of cells enter into dying state as consequence to apoptosis regulation. This type of molecular mechanism seems to result from diversified pathways and has proved imperative in the preservation of health and hindrance of cancer. Cells undergoing oncogenic stresses, may find themselves mislead into uncontrollable cells division or DNA damages, that may lead to apoptosis. Flowingly, the selective
elimination of cells through apoptosis is a deliberate process to keep cancer cell growth at bay.

Exceptional sources of bioactive compounds have been reported from plant consumption exerting beneficial remedies in health restoration. A percentage of 50% to 60% of cancer patients have been resorting to plant derivatives either directly or accompanied by modern regimens like chemotherapy or radiation therapy [20]. Such compounds include curcumin (turmeric), genistein (soybean), tea polyphenols (green tea) and diallyl sulphide (garlic) [21]. For example, the apigenin, a flavones present in vegetables was exhibited to induce apoptosis in human colon cancer [22] curcumin was shown to trigger apoptosis in cancer cells through NF-κB interference without harming the healthy cells [23] and crocetin, deriving from saffron, inhibits cancer cells by affecting nucleic acid synthesis, inducing apoptosis, all at the same time preventing growth factor signalling pathways [20]. These phytochemicals in short, had been described to inhibit progression of various cancers. Therefore, current research was conducted to provide biochemical evidence on apoptotic cell death activities of PLME in detail by multi parametric flow cytometric analysis and biochemical assay of functional events associated with cell apoptosis in HeLa cells.

4.1. Flow cytometry analysis of apoptosis in HeLa cells

In this study flow cytometry method was used for apoptosis detection in HeLa cells treated with PLME. As depicted in the top panel of Fig. 2, the percentage of cells that underwent each of the apoptotic changes in 24 h strictly depended on the concentration of PLME used. The scatter plot graph visibly shows the progression from early to late apoptosis as indicated by the concentration-dependent accumulation of the late apoptotic cells. This observation fall in accordance with [24] where the aqueous fraction of Nephelium ramboutan-ake is gradually shown triggering the shift in percentages towards late apoptotic effect in HT-29 (human colorectal cancer) as the concentrations increase from 25 to 200 μg/mL.

The PLME treatment at IC50 concentration showed the presence of apoptotic cells (early and late apoptosis) at 50.0% while 49.37% represents viable cells. This result justified that the median reading of the IC50 concentration obtained from MTT and CyQUANT [8], which inhibited approximately 50% of the cells. Roy et al. [25] reported that ethanolic extract of Moringa oleifera root bark (EMORB) with IC50 of 30.22 μg/mL and 28.29 μg/mL (obtained by MTT), only tended to generate percentages of apoptosis at 10.9% and 18.6% for U937 and K562 (human leukaemia cell lines) respectively. In another study, cycloartane triterpenoid from Commiphora myrrha, exhibited a cytotoxicity activity at IC50 concentration (9.6 μM) against PC3 cells (human prostatic cancer) by generating apoptotic cells at 20.9% and a 1.5-fold of this IC50 (14.4 μM) concentration produced a percentage of 40.7% [26] apoptotic cells. It can be concluded that PLME with the inhibition concentration at 50% is able to exert a higher percentage of apoptotic cells compared to other studies with various phytochemicals of IC50 concentration.

Notably, the necrotic cell population remained low throughout Fig. 2A. A study reported that the effect of a certain death stimulus is common in displaying the continuum of apoptosis to necrosis [27]. Several drugs have induced apoptosis at lower concentrations and necrosis at higher concentrations. This condition is a consequence of to the absence of phagocytic cells in the cell culture, which may be the reason for the presence of necrotic characteristics owing to the lack of cellular energy and plasma membrane integrity. This is then referred to as secondary necrosis cell death [27]. As the concentration of PLME increased from half IC50 to double IC50 more 85% in V [28] was detected binding to PS on the outer leaflet denoting the occurrence of apoptosis in HeLa cells at all the PLME concentrations tested. These findings confirmed that apoptosis might be the mechanism by which the PLME triggers cell death in HeLa cells. Polyphenolic compounds from medicinal plants have been shown to inhibit or attenuate the initiation, progression and spread of cancers in cells in vitro and in animals in vivo [28]. Hence, the polyphenolic compound(s) in the methanol extract are responsible for the effects of PLME against HeLa cells. Our previous studies have found that the leaf extract of P. longifolia exhibited good antioxidant activity because of the high content of polyphenols and flavonoids (quercetin, rutin, narcissin,isorhamnetin, and kaempferol) in P. longifolia [6]. Thus, in this study, we predict that the polyphenol and flavonoid molecules may also play an important role in the observed cytotoxicity of PLME against HeLa cells. Furthermore, the noncancerous Vero cells tested with PLME exhibited no cytotoxicity with the IC50 value of 51.07 μg/mL at 24 h by using MTT assay [8]. Moreover, an acute oral toxicity study revealed that PLME was safe after oral administration as a single dose to female albino Wistar rats with up to 5000 mg/kg body weight [6]. The results of the in vitro and in vivo tests demonstrated that PLME was devoid of a significant toxicity effect under experimental conditions and an ideal candidate for developing a nontoxic anticancer agent.

4.2. Flow cytometry analysis of the PLME effect on cell cycle distribution

The cellular DNA content was investigated by introducing PI, which has an affinity towards DNA followed by FACS analysis of the fluorescence intensity of single cells to differentiate cells at G0/G1, S and G2/M phases. Referring to Fig. 3, the G0/G1 was arrested at the percentage of 64.21 ± 1.13%, 66.11 ± 1.11%, and 86.96 ± 0.31% in accordance with the treatment of PLME of half IC50, IC50, and double IC50 concentration. Together with that, the G2/M phase was also observed to initially demonstrate small percentages of growth arrest at 3.66 ± 1.82% (half IC50), and 2.31 ± 1.43% (IC50), and with the highest concentration (double IC50), the percentage was observed to be 22.2 ± 0.85%. Hence, it is conclusive that HeLa cells can be arrested in the G1 phase by PLME, either during the G0/G1 transition or during G1 of the normal cell cycle. However, the S phase was relatively transient, and cells were seen to migrate to the G2/M phase after the increase in the treatment in a dose-
dependent manner. In contrast to the S phase, the G2/M phase for the DNA damage checkpoint proved to be more permanent. Hence, over the increase of PLME concentration, cells that were initially arrested in the G0/G1 phase were also seen to accumulate during the G2/M phase.

The hindering of the cell cycle at these points not only prevents the cells from proliferating but also provides cells with time for DNA repair. If the damage is beyond repair, then apoptosis is triggered through p53 dependent or independent pathways [29]. Targeting cells to be arrested at G2/M phases is considered to be an intriguing factor in cancer therapy since cancer cell sensitivity has been attributed to DNA damaging chemotherapeutic agents [30–32]. Similar results were obtained for a dietary compound 3,3'-diindolylmethane (DIM), which was found to arrest HT-29 human colon cancer cells during the G0/G1 and G2/M phases. This shows that DIM inhibits HT-29 cell growth via the induction of cell cycle

![Image](https://example.com/image.png)
and apoptosis in a dose-dependent manner (10–30 μmol/L) [33]. Another study also implicated silibinin (flavonoid from fruits) and silymarin (flavonolignan complex) in the inhibition of the proliferation of PC3, human prostate cancer cells. Cell death was induced due to the arrest in the G0/G1 and G2/M phases in a dose-dependent fashion (50–100 μg/mL) [34]. While most of the potent phytochemicals were reported to induce G2/M [35–37] through various cell cycle signalling pathways, the PLME has a unique ability to exert growth arrest at two different checkpoints.

4.3. Flow cytometry analysis of the PLME effect on mitochondria membrane potential ($\Delta$$\Psi$mem)

The apoptotic intrinsic pathway is closely bound to the stimulus regulated from the mitochondria [38,39]. Moreover, the findings

![Flow cytometry analysis of the PLME effect on mitochondria membrane potential ($\Delta$$\Psi$mem)](image)
revealed that the mitochondrial activity in cancer cells is dissimilar to that of normal cells due to the failure of apoptosis, which, in turn, has been shown to have an impact with chemo and radioresistance in cancer therapy. Thus, a strategy was constructed to introduce the malfunction of mitochondria in cancer cells to manage their growth and dispersion. The key element to achieving such a goal is to induce a loss of ΔΨm to eradicate cancer [40,41].

Based on Fig. 4, exposure of PLME, even at the lowest concentration, is able to exert a large shift in the loss of ΔΨm. In brief, as the concentration of PLME increased, a gradual decrease in ΔΨm was demonstrated. This was evident when the JC-1 monomer represented by green fluorescence increased from 86.43 ± 0.87% (half IC50) to 98.27 ± 1.12% (IC50), and, finally, to 98.53 ± 0.76% (double IC50). Other natural compounds, such as heteroemnin (HET), a known sesterterpenoid extracted from the sponge Hippospongia sp., was also found to induce apoptosis in T24 bladder carcinoma cells via the loss of ΔΨm. Elevated percentages of 36.6%, 78.6%, 85.6% and 95.3% of green monomers were observed with the corresponding HET concentrations of 0.1 μg/mL, 0.2 μg/mL, 0.3 μg/mL and 0.4 μg/mL [42]. The methanol extract of Dorstenia psilurus (medicinal plant) exhibited a loss of ΔΨm in human promyelocytic leukaemia (HL-60) cells. The percentages of depolarised ΔΨm were observed to increase at 23.03%, 51.32% and 88.13%, respectively, which corresponded to the crude extract concentration of 20 μg/mL, 50 μg/mL and 100 μg/mL [43]. Based on this evidence, PLME is proven to be a better candidate in exerting the loss of ΔΨm compared to HET and D. psilurus extract in a dose-dependent manner.

Moreover, other phytochemical, such as quercetin, inhibited HeLa cell proliferation by arresting the cell cycle in the G2/M phase while triggering apoptosis through the disruption of ΔΨm [44]. In another study, α-mangostin, a compound found in mangosteen, was shown to induce apoptosis in human oral squamous cell carcinoma (OSCC) cells by arresting the cell cycle in the G0/G1 phase, which was accompanied by dysregulation of the mitochondrial function [45]. Capsaicin, a major constituent of chilli peppers, triggers cell cycle arrest in the G2/M phase and activates apoptosis through dissipation of the mitochondrial membrane potential in KB cancer cells [46]. Conclusively, PLME, relative to quercetin, α-mangostin and capsaicin, has been proven to cause apoptosis in HeLa cells through the mitochondrial intrinsic pathway.

4.4. PLME effect on ROS generation

Recent studies indicated that many chemotherapeutic agents are capable of killing tumour cells by regulating oxidative stress prior to inducing cell death [47]. Fig. 5 displays the interesting role of PLME in evoking antioxidant and pro-oxidant in HeLa cells depending on the effect of the concentration. At half IC50, PLME is shown to reduce the level of ROS significantly after 24 h. The PLME at the lowest concentration seems to function as an antioxidant by scavenging ROS in the HeLa cells, and, with the increasing concentration, induces apoptosis through pro-oxidant action. This ability proves that PLME is capable of modulating the redox state of HeLa cells.

In vitro studies have reflected that the cytoprotective activity of phytochemicals, such as polyphenols, are effective in preventing cell death by causing oxidative stress [48–50]. However, they have also been proven to cause pro-oxidant activities under specific conditions, such as at higher amounts or in the presence of metal ions [51–53]. The phytochemical action in exerting pro-oxidant or antioxidant is completely dependent on their concentration. In conjunction with that, collective studies have emerged that draw attention to the phytochemical-induced pro-oxidative responses by antioxidants, such as quercetin, kaempferol, epicatechin, epigallocatechin-3-gallate (EGCG) and gallic acid [54–56]. Quercetin, at high concentration (50 μM), generated ROS in isolated mitochondria taken from human colon tumour (HCT116) cells [56]. In a different study, quercetin, at lower concentrations (0.1–20 μM), was observed to display antioxidant activity and cell proliferation, whereas a higher concentration was found to inhibit the cell viability and growth of adenocarcinomic human alveolar basal epithelial (A549) cells. Other findings have also demonstrated that, at low concentrations (10–25 μM), quercetin and fisetin preserved the rat hepatoma cell line (H4IE) from the injurious effect of ROS-induced cytotoxicity, while increased concentrations (50–250 μM) caused a decrease in apoptosis and the cell viability [57].

Many studies have revealed that a number of chemotherapeutic agents cause cell death through ROS escalation [58,59]. It is understood that cancer cells utilised ROS-mediated signalling to prolong their survival ability; if this theory is correct, then the introduction of any agent with the aptitude to increase or decrease ROS may compel a cancer cell to go beyond its threshold on lipid peroxidation, DNA damage, and protein oxidation, thereby succumbing to death.

4.5. DNA fragmentation as induced by PLME in HeLa cells

Lately, comet assay has received interest in scientific research due to its potential as a suitable tool for the determination of genotoxicity [60,61]. Hence, in this study comet assay was used to study DNA fragmentation induced by PLME in HeLa cells. PLME induced DNA damage in HeLa cells in a dose-dependent manner. As shown in Fig. 6A, the DNA damage in the HeLa cells, was found to increase in cell numbers parallel to the increase in PLME concentration. This observation is in accordance with the investigation by Chen et al. [62] on Bezielle flavonoids. Bezielle, a mixture of botanical extracts, has selective anti-tumour activity on breast cancer cells (MDA-MB-231) through the induction of ROS in tumour cells, which lead to heavy DNA damage. The flavonoids obtained from Bezielle indicated significant DNA damage at concentrations of 5 μg/mL and higher in a dose-dependent manner. This correlates with the increased percentages of cell comets and ROS level. The treatment of green tea polyphenols (GTP) on human melanoma cell lines revealed a significant increase in the length of comet compared to non-GTP treated cells. The data further disclosed the potential of GTP in inducing DNA damage in a concentration-dependent manner (0, 20, 40, and 60 μg/mL) for 48 h in melanoma cells, which resulted in the suppression of the cell viability of melanoma cells [63]. The induction of Tinospora cordifolia (TCE) extract through increasing concentrations on HeLa cells revealed greater DNA damage in terms of concentration-dependent, which was expressed as olive tail moment (OTM) [64]. Although OTM appeared to be a statistically significant measurement, the inter-laboratory comparison of the results appeared to be complicated for this parameter, and, hence, was omitted in this present study [60].

Complications in comet results arose since there is no simple connection with the degree of DNA damage caused by this type of chemical. Every cytotoxic drug exerts a variety of DNA damage breaks in relation to their biological impact on the cells [65]. Generally, the cause of DNA damage cannot be directed to the genotoxicity alone as dysfunctional mitochondria or membrane damage may also impact DNA fragmentation via apoptosis or necrosis. In such circumstances, the comet assay results should be correlated with cytotoxicity to gain a direct perspective between the DNA damage and cell death. However, in this study the flow cytometry analysis of apoptosis in HeLa treated with PLME displayed apoptotic cell death, which also establishes a possible link with DNA fragmentation.
The exact mechanism of induction of DNA damage by PLME is not known. The increased DNA damage may not be ascribed to a single mechanism, and several putative mechanisms may be responsible for the induction of DNA damage by PLME. Previously, PLME was revealed to induce free radicals and generate ROS causing damage to cellular DNA. These free radicals might have caused damage to DNA by hydrolysis, oxidation and electrophilic attack. As such, a study conducted with daidzein, a phenolic compound found in soya beans, increased the tail length of BEL-7402, hepatoma carcinoma cells when exposed to increasing concentrations of 30 and 60 μM. Daidzein was reported to increase the levels of ROS while inducing a decrease in ΔΨm and inhibiting cell cycle progression in the G2/M phase [66]. In the present study, cells with damaged DNA were shown to possess increased migration of DNA fragments (comet tail) from the nucleus (comet head), as evidenced by the increase in comet number, tail length, tail moment, percentage tail DNA, which may also be a feature of DNA fragmentation associated with apoptotic cell death [67,68].

4.6. Analysis of the apoptotic proteins detection from RayBio array

Several studies employing the RayBio microarray have been successful in the investigation of apoptosis of cell lines through phytochemical elicitation [15,69,70]. The PLME at IC50 induced HeLa cells revealed an increase in BAX, BAD, caspase-3, cytochrome c, p53, p21 and p27, while the BCL-2 and BCL-w were found to decrease significantly.

The members of BCL-2 can be classified into three groups based on the region of BCL-2 homology (BH domains): (1) class that prevents apoptosis constituted by BCL-2, BCL-xl, BCL-w, and BCL-b; (2) class that stimulates apoptosis composed of BAX, BAK and BOK; and (3) a class with conserved BH-3 domain that has the ability to influence anti-apoptotic BCL-2 members into causing apoptosis, such as BAD, BIK, BIM, NOXA and PUMA. The BAX has been shown in this study to increase after PLME treatment. In addition, this pro-apoptotic protein, together with other BAX-like proteins (BAK), were reported as being the factors responsible for the outer mitochondrial membrane (OMM) permeabilization and the discharge of cytochrome c, a molecule of apoptogenic that activates the caspase cascade [71–73]. BAX exists in the cell cytosol [74], and only with the initiation of apoptosis travels to the mitochondria [69,75]. The increase in BAX coincides with the loss of ΔΨm, while the increase in cytochrome c and caspase-3 are in agreement with the findings of this study.

The increase in BAX has been equated with the increase in cytochrome c. Cytochrome c is a complex protein localised within ETC, where it anchors itself to cardiolipin, a component found in the inner mitochondrial membrane. Stimulation of early apoptosis will generate massive ROS, which will then oxidise the cytochrome c-cardiolipin complex causing it to detach and expel cytosol through the mitochondrial membrane pores [76]. The IP3 receptor (IP3R) on the endoplasmic reticulum (ER) reacts with the initial release of cytochrome c causing it to discharge calcium (Ca2+). The accumulation of Ca2+ triggers a further release of cytochrome c [77]. The subsequent release of cytochrome c will initiate the activation of caspase-9, which will initiate a cascade of events to promote apoptosis. Caspase-3 is known to be an executioner caspase, and, upon the release of cytochrome c, sets a chain of reactions that leads to the annihilation of cells from within [78]. The presence of caspase was also indicated in HeLa cells treated with isothiocyanate, a compound found in the ‘cabbage-family’ [79].

Cancer cells were postulated to exist continuously by directing various mechanisms to inhibit or evade apoptosis [9,80]. As such, one mechanism is to express a massive amount of anti-apoptotic BCL-2 protein members. In that regard, the functions of BCL-2 and BCL-w (positioned at the outer mitochondrial membrane) contradict those proteins mentioned above, as they inhibit the apoptotic mechanism by blocking the role of BAX and BAK in the permeabilization of mitochondrial membrane to release cytochrome c and the sequential activation of caspase activity [81,82]. Van Delft et al. [83] reported that a BH3 mimic inhibited the progression of BCL-2 and BCL-w through the induction of BAX/BAK-mediated apoptosis, as demonstrated in the mouse lymphoma model. Clearly, these anti-apoptotic proteins assert the possibility of being restrained for a potential lead to apoptosis in cancer cells.

BAD is associated with promoting apoptosis by binding to BCL-2, BCL-w and BCL-xl and causing inhibition; hence, resuming BAX/BAK-mediated apoptosis [84–86]. When apoptosis is not required, the BAD is kept inactive and at bay through serine-threonine kinases phosphorylation [87,88], which prevents the action mechanism of cytochrome c [87]. The phosphorylation binds BAD to 14–3–3 protein where the complex withdraws to cytosol [89,90], whereas dephosphorylation unleashes BAD, carrying it to the mitochondria to block the actions of BCL-2, BCL-xl and BCL-w.

This clearly indicates that PLME-induced HeLa cell death is associated with a change in p53, p21 and p27 gene expression, and is also accompanied by an overexpression of BAX, BAD, caspase-3 and an increase of cytochrome c acting in accordance to promote cell death by apoptosis. Apoptosis is frequently the end point of cell fate, and, therefore, a process that eventuates cell death should diminish the proliferation of stimuli. Indeed, in this study, apoptotic cell death is preceded by the arrest of the cell cycle and accumulation of cells in the G0/G1 phase and G2/M phase. In that the up-regulation of BAX has been insinuated in an early study, which implicates the activation and induction of Cdk and caspases through the breakdown of p27 inhibitor [91], it is only rational to examine the presence of p53, p21 and p27 in cell cycle and apoptosis progression triggered by PLME in HeLa cells.

The p53 has a widespread reputation as a tumour suppressor and its anticancer functions range from operating during apoptosis to causing genomic instability and prevention of angiogenesis. This tumour suppressor gene has several mechanisms that cause cell growth arrest [92] and programmed cell death induction [93] during DNA damage [94]. ML-1 leukaemia cells subjected to ionising radiation were shown to arrest growth in the G0/G1 phase as the outcome of p53 following DNA damage [95]. Relatedly, treated human glioblastoma cells with dexamethasone, also showed an increase in p53 that arrested those cells in G0/G1 [96]. In addition, reports have been published indicating that p53 is a transcriptional activator with the tendency to regulate genes, particularly p21 [97,98]. The p21 (p21Waf1 or p21Cip1) is a cyclin-dependent kinase inhibitor that hinders the action of Cdk1 and Cdk2 from regulating the cell cycle [97]. The fact that p21Waf1 inhibits both Cdk, illustrates the potential for the G0/G1 and G2/M phases to be arrested. Studying the level of p53 in MDAH041 human fibroblasts confirmed that p53 in mediates growth arrest in both the G0/G1 and G2/M phases with a considerable induction of p21Waf1 [92]. The detection of p53 and p21 in the PLME treated cells was observed to be remarkable with the fold change of 3.700 and 3.217, respectively, which may be the conceivable factor for the HeLa cells arrest in the G0/G1 and G2/M phases. Apart from p53 and p21, proteins, such as p27Kip1 were also investigated to explore their connection to cell cycle regulation and apoptosis. The p27Kip1 (p27) warrants the well-organised advancement of the cell cycle, which binds to cyclin D, E, A, and B-dependent kinase for negative regulation [99,100]. Overexpression of p27Kip1 was insinuated with G0/G1 phase arrest [101,102].

Several phytochemical compounds were reported to exert an apoptotic effect in cancer cells, and one such analogously studied is the curcumin in HT-29 colorectal cancer cells. The cell viability
reduction along with higher expression of cytochrome c, caspase-3, BAX and BAD were observed. On the other hand, expressions of BCL-2, BCL-xL and survival were also noted to decrease in accordance with 10–80 μmol/L curcumin [103]. In other findings, the sulphoraphane was also found to cause apoptosis in HT-29 cells. The treated cells revealed the display of PS on the outer membrane leaflet, chromatin condensation and expressions of pro-apoptotic proteins. The increase of BAX and cytochrome c was implicated while levels of p53 were found to be unchanged. In conclusion, the pro-apoptotic proteins illicit through the exposure of PLME not only exposed its relevance to the apoptosis mechanism but illustrated the interconnected networks in respect of the loss of mitochondrial integrity, generation of ROS, DNA damage and cell cycle arrest at G0/G1 and G2/M in HeLa cells.

5. Conclusion

As shown in Fig. 8, the treatment of PLME with different concentrations of HeLa cells resulted in a concentration-dependent increase in PS exposure on the outer leaflet of the mitochondrial membrane, cell cycle arrest, loss of ΔΨm, generation of ROS and DNA fragmentation. The PLME may have caused oxidative stress by increasing the formation of free radicals and ROS generation. The effective cell killing by PLME may be postulated as leading to the induction of DNA damage and the loss of DNA repair capacity. The IC_{50} concentration of PLME was revealed to increase the level of pro-apoptotic BAX, BAD, caspase-3, p53 and p21 significantly, while causing a decrease in the expression of anti-apoptotic proteins, BCL-2 and BCL-W. In conclusion, the PLME induced p53 mediated apoptosis, cell cycle arrest and mitochondrial potential depolarization by modulating the redox status in the HeLa cells.

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References


Fig. 8. Schematic image representing the postulated mechanism of PLME inducing apoptosis in HeLa cells.

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Conflict of interest

The authors declare that they have no conflict of interest.


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