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Induction of Apoptosis and Cell Cycle Arrest in Human Breast Cancer Cells T-47D and MDA-MB-453 Treated with *Polyalthia longifolia* Methanolic Extract

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ABSTRACT

Polyalthia longifolia cv. pendula (Family Annonaceae) contains active phytochemical moieties in various parts of the plant providing a valuable source for curative strategies in ailments and diseases. In the present study, the efficacy of Polyalthia longifolia leaves Methanolic Extract (PLME) was validated against two different human breast cancer cell lines-invasive ductal carcinoma cells-T-47D and adenocarcinoma cells-MDA-MB-453 cells. This reinforces the first report emphasizing the induction of apoptosis in PLME treated MDA-MB-453 cells. After 24 hours, the IC50 value of PLME was observed as 23.58 ± 3 µg/mL and 17.1 ± 1.2 µg/mL in T-47D and MDA-MB-453 cells, respectively. The early apoptotic population in PLME (20 µg/ml) treated T-47D cells was found to be 24.64% by the Annexin-V method. In MDA-MB-453 cells, 50.4% and 66.1% early apoptosis was recorded in 20 µg/ml and 40 µg/ml of PLME treated cells, respectively. The dose-dependent cell cycle arrest was observed in PLME treated T-47D and MDA-MB-453 cells. The number of cells in the G2/M phase increased, while that in the G0/G1 phase decreased in PLME treated cells. The increased concentration of PLME (40 µg/ml) significantly increases the arrest of T-47D cells in the G2/M phase (28.9%). In T-47D cells, PLME treatment resulted in a highly significant ($p<0.0001$ compared to untreated cells) down-regulation of BCL2. The increase in the concentration of PLME resulted in significantly increased expression of BAX and BAK1. In the current findings, the upregulation of BAK1 and BAX, cell cycle arrest, and Phosphatidylserine on the membrane of T-47D and MDA-MB-453 cells confirm apoptosis in presence of PLME.

Keywords: Polyalthia longifolia cv. Pendula, T-47D and MDA-MB-453 cells, BAK1 and BAX, Annexin-V

INTRODUCTION

Polyalthia longifolia cv. *pendula* (Family *Annonaceae*), found in India are locally known as "Ashoka". The genus *Polyalthia* includes about 120 species spread across three continents-Africa, Asia, and Australia [1]. Plant materials act as sources for effective concurrent administration of extracts in therapeutics, thus enhancing its applications as folklore and indigenous medicines. In Ayurveda, herbal preparations of *P. longifolia* have been mainly used to treat duodenal ulcers while decoctions of the plant have been used in the treatment of fever, diabetes, and skin diseases in various traditional medicine systems [2]. The *Polyalthia* genus contains clerodane diterpenoids and alkaloids in various parts of the plant [3-5]. The bark is anthelmintic and proved effective in the treatment of fever, skin disease, diabetes, hypertension, helminthiasis, and vitiated conditions of *vata and pitta* [6]. The bark and leaves extracts are beneficial as therapeutic properties which exhibit antimicrobial, anticancer, antiproliferative, antiulcer, hypoglycaemic, and hypotensive action [1]. The *P. longifolia* leaves extract inhibited cell proliferation of various human cancer cell lines [7-11]. The recent studies emphasized different gene mutations in progenitor cells causing diversity in subtypes of breast cancer in humans. Conventional chemotherapy, radiotherapy, and surgery depicted limited effects on breast tumors growth at primary and secondary sites. The diversity of natural products extracted from plant sources may provide an alternative therapy to prevent side effects in cancer patients. In this study, *Polyalthia longifolia* leaves Methanolic Extract (PLME) was investigated for its efficacy against two different human breast cancer cell lines-invasive ductal carcinoma cells-T-47D and adenocarcinoma cells-MDA-MB-453 cells. The cell cycle arrest, Annexin V- FITC Assay, expression of pro and anti-apoptotic genes were analysed in PLME treated cells. This portrays as the first report of apoptosis in PLME treated MDA-MB-453 cells.

MATERIALS AND METHODS

Collection of *P. longifolia* leaves and Preparation of Methanolic Extract of Leaves

Polyalthia longifolia leaves were collected from the local surroundings of Aurangabad city, India. The methanolic extract was prepared by using the method described previously with minor modifications [12,13]. Briefly, the fresh leaves were separately cut to small pieces and completely air-dried in shadow for 7 days. Each part of shed dried leaves was mixed with five volumes of the methanol and extracted by using the Soxhlet method [13]. The extract was labeled as PLME (*Polyalthia longifolia* Methanolic Extract). The extracts were kept at -20°C for conducting further experiments.

Cell Lines

The human breast carcinoma cell lines, T-47D and MDA-MB-453 used in the present study were procured from National Centre for Cell Sciences (NCCS), Pune, India. The T-47D cells were maintained in complete growth medium RPMI-1640 (Thermo Inc, USA) supplemented with 10% heat-inactivated fetal bovine serum (Himedia, India), 0.2 Units/ml bovine insulin, 1% penicillin, and streptomycin. For MDA-MB-453, L-15 medium (Himedia, India) supplemented with 10% heat-inactivated fetal bovine serum (Himedia, India) was used. The cells were further sub-cultured and incubated in a CO_2 incubator at 37°C in a humidified atmosphere with 5% CO_2. At 70%-85% confluency, the cells were trypsinized with 0.25% TPVG (HiMedia, India), counted, and aliquot at the desired density for growth assays. The cells were freshly grown by diluting the stock with phosphate-buffered saline (PBS, pH 7.2), which was applied in all the experimentation.

Cell Proliferation Assay

MTS assay is a colorimetric method equipped for sensitive quantification of viable cells which was applied for the cell proliferation assay. The NAD(P)H-dependent dehydrogenase enzymes reduce MTS tetrazolium compound to form a soluble formazan product [14]. The viability of PLME treated T-47D and MDA-MB-453 cells was determined with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). The protocol was followed according to the manufacturer's instructions with minor modifications. Briefly, the cells (~1×10^3) were plated in 100 µL cell culture (RPMI-1640 or L-15) medium in 96 well plate and (0, 0.25, 5, 10, 20, 40 and 80) µg/mL PLME was added to each well. Each concentration of PLME was repeated three times. The cell viability was determined 24 h and 48 h after incubation in a CO_2 incubator at 37°C. The plates were incubated for 4 h, after the addition of MTS (5 mg/mL in PBS) to each well of the plate. The absorbance was recorded at 490 nm using a 96 well Multiscan Ascent (Thermo Inc. USA). The inhibitory effect of PLME on cell growth was assessed as percent cell viability, where the cells without treatment were considered 100% viable.

Determination of Phosphatidylserine on Membranes of PLME Treated Cells

The Annexin V-FITC Apoptosis Detection Kit (Thermo Inc. USA.) was utilized for the analysis of phosphatidylserine exposure according to the manufacturer's instructions. Briefly, the cells were plated at ~1×10^5 cells in each well of 6 well plates in 500 µL complete cell culture medium with 10% v/v FBS and (20, 40) µg/ml PLME was added to each well. After 24 h, the cells were harvested, washed with cold PBS, and re-suspended in 200 µL Annexin binding buffer (50 mM HEPES, 700 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Annexin V-FITC 5 µL and 1 µL PI (100 µg/ml) were then added to 100 µL cells suspensions. After incubation of 15 min at room temperature, 400 µL of the Annexin binding buffer was added before analysis by Attune flow cytometer (Thermo Inc. USA), and further calculated by Attune cytometric software v2.1 (Thermo Inc. USA).

Inhibition of Cell Cycle in PLME Treated Cells

1×10^5 cells of each cell line were plated in six-well plates and allowed to adhere overnight before serum starvation. After 24 hours of serum starvation, PLME was added. The spent media were collected and spun at 1000 × g for 5 min to collect detached cells or floaters, which were combined with adherent cells for cell cycle distribution analyses by flow cytometry. The cells were washed twice with PBS and fixed in 70% (v/v) ice-cold ethanol at 4°C for 24 h. 50 µL of RNase A solution (100 µg/mL in PBS) was added to the cells. The fixed cells were stained with propidium iodide (Thermo Inc. USA). The samples were then analyzed in an Attune flow cytometer (Thermo Inc. USA).

Downregulation of Anti-Apoptotic Genes in PLME Treated Cells

Expression levels of RNA transcripts were quantitated in PLME treated and untreated cells. The cells were plated at ~1 × 10^4 cells in each well of 12 well plates in 300 µL of complete cell culture medium with 10% v/v FBS and 20 µg/mL PLME was added to each well. Total RNA from T-47D and MDA-MB-453 cells were isolated using iScript RT-qPCR sample preparation reagent (Biorad, USA). SYBR green assay (Biorad, USA) was used for the Real-time quantization of the RNA. After RNA isolation, RNA to CT kit (Thermo Inc.) was used for Real-Time PCR. The primers used were previously described and real-time PCR was performed according to the kits manufacturer's instructions (Thermo, USA) [13]. For each single-well amplification reaction, a threshold cycle (CT) was observed in the exponential phase of amplification and the quantization of relative expression levels was achieved using standard curves for both target and endogenous controls.

Statistical Analysis

GraphPad Prism 6.0 software was used for the interpretation of the statistical analysis. Each experiment was performed in triplicates. The statistical differences were evaluated by students' two-tailed t-tests and the Analysis of Variance (ANOVA) test which was considered significant at $p<0.05$.

RESULTS

In the present findings, the breast cancer cells were found unhealthy after exposure to PLME on human breast cancer cells, T-47D and MDA-MB-453. The effect of PLME induces apoptosis in these cells and is substantiated by using an MTS assay.

MTS Assay

The two breast cancer cells T-47D and MDA-MB-453 were treated with PLME for the analysis of the antiproliferative effect of PLME on human breast cancer cells and its efficacy was evaluated by MTS assay (Figure 1). The IC50 value of PLME in T-47 D after 24 h is, low i.e., 23.58 ± 3 µg/mL (Figure 1a). The IC 50 value of PLME in T-47D cells after 48 h has been found 17.59 ± 1.9 µg/mL (Figure 1b). As shown in Figures 1a-1d a dose-dependent increase in the death of cancer cells was observed with increasing concentration of the PLME. The IC50 was found to be 17.1 ± 1.2 µg/mL in MDA-MB-453 cells (Figure 1c). The cell viability was found to decrease after 48 hours in both the cell lines (Figure 1b and Figure 1d). Significantly decreased cell viability was found in PLME treated T-47D and MDA-MB-453 cells ($p<0.0001$ compared to untreated control).

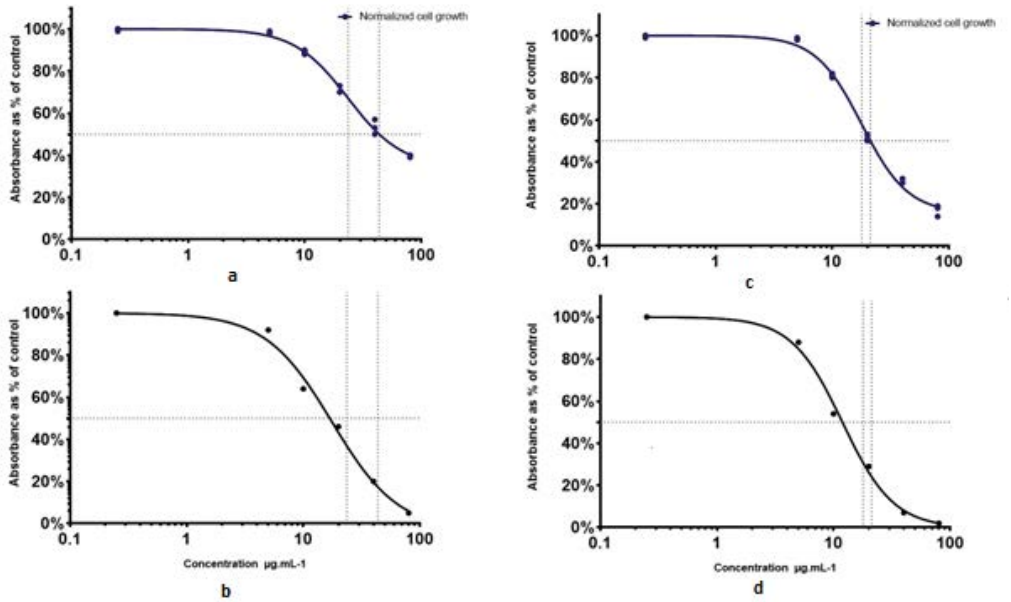

Figure 1 Evaluation of the viability of PLME treated Human breast carcinoma cells-T-47D and MDA-MB-453 cells.

T-47D and MDA MB 453 cells were treated with (0, 0.25, 5, 10, 20, 40 and 80) µgmL^{-1} PLME; Where, a): T-47D cells treated with PLME for 24 h; b): MDA-MB-453 cells treated with PLME for 24 h; c): T-47D cells treated with PLME for 48 h; d): MDA-MB-453 cells treated with PLME for 48 h. Cell viability was monitored by MTT assay. The percentage of viability was calculated as the following formula: (viable cells) %= (OD of drug-treated sample/ OD of the untreated sample) × 100. Measurements were made in triplicate, and standard bars represent the standard deviation. The Student's two-tailed t-test was used to determine the statistical significance of the differences between untreated cells and cells treated with the various concentrations of PLME

Analysis of Phosphatidylserine on T-47D and MDA-MB-453 Cell Membrane

Annexin-V staining method was applied for the analysis of the morphological changes during induction of apoptosis. Morphological changes indicated the induction of apoptosis in the breast cancer cells-T-47D and MDA-MB-453 (Figure 2). Annexin V stained cells by flow cytometry (Figures 2a-2d) confirms the presence of early and late apoptosis (Figures 2a-2d). The healthy viable cells population was (93%) in untreated cells. In PLME (20 µg/ml) treated T-47D cells, 24.64% population was early apoptotic events (Figure 2a). The cell population in late apoptosis was recorded as 0.1% to 0.24% in T-47D cells treated with PLME concentrations of 20 µg/ml and 40 µg/ml, respectively. The early apoptosis population was observed to be increased (29.14%) in 40 µg/ml PLME treated T-47D cells (Figure 2b). In MDA-MB-453 cells, the PLME treatment significantly increases the apoptotic cells population, 50.4% (Figure 2c) and 66.1% (Figure 2d) in 20 µg/ml and 40 µg/ml of PLME, respectively. The growth inhibition leads to the induction of apoptosis in PLME treated MDA-MB-453 and T-47D cells.

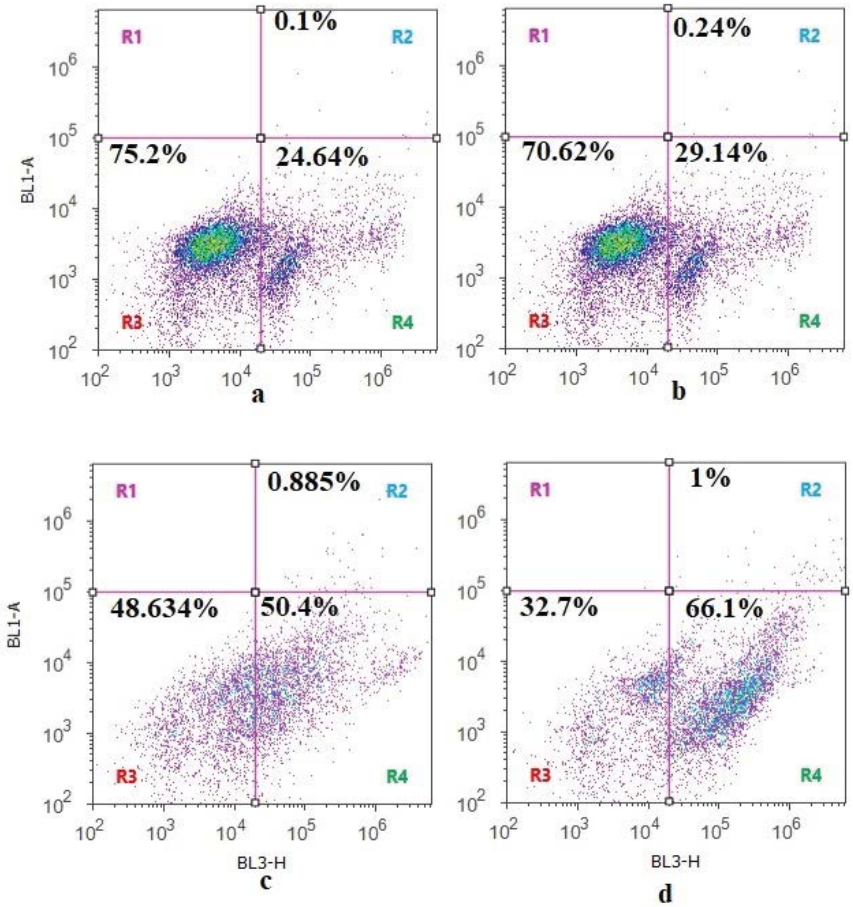

Figure 2 Detection of membrane proteins on PLME treated cells. Apoptosis in T-47D and MDA-MB-453 cells was assessed after 24 h of treatment with PLME by Annexin V, Alexa Fluor® 568 conjugate /PI binding and measured by flow cytometry; Where; a): T-47D cells with 20 µg/ml PLME; b): T-47D cells with 40 µg/ml PLME; c): MDA-MB-453 cells with 20 µg/ml PLME; (d): MDA-MB-453 cells with 40 µg/ml PLME

Effect of PLME on Cell Cycle of T-47D and MDA-MB-453

The effects of PLME on the cell cycle of T-47D and MDA-MB-453 cells were evaluated by using PLME treated and untreated cells. The Flow cytometry was used for quantification of the cell cycle distribution under treatment with PLME to determine whether PLME induced apoptosis was related to arrest of cell cycle progression in breast cancer cells indicating concomitant analysis of apoptosis (Figure 3). The number of cells in the G2/M phase increased, while that in the G0/G1 phase decreased in PLME treated cells (Figures 3a-3f). The G2/M phase population was reported as 20.9% in PLME (20 µg/ml) treated T-47D cells (Figure 3b). The increased concentration of PLME (40 µg/ml) significantly increases the arrest of T-47D cells in the G2/M phase (28.9%) (Figure 3c). The reduced population of G0/G1 was found in PLME treated cells (Figures 3a-3f). In MDA-MB-453 cells, the most significant effect of PLME on the cell cycle was recorded (Figures 3d-3f). 20 µg/mL of PLME inhibits the MDA-MB-453 cells in G2/M phases (47.5%) (Figure 3e). The increased concentration has reduced the G1 and S phase cell population and the cell cycle was found arrested in the G2/M phase (56%) (Figure 3f). This experimental finding implies that PLME induced apoptosis in breast cancer cells *via.* G2/M phase arrest. The G1 and S phase MDA-MB-453 cells were 93% in untreated wells and decreased to 47% and 33% in cells populations treated with 20 µg/mL and 40 µg/mL of PLME, respectively (Figures 3d-3f). The dose-dependent cell cycle halt was observed in PLME treated T-47D and MDA-MB-453 cells.

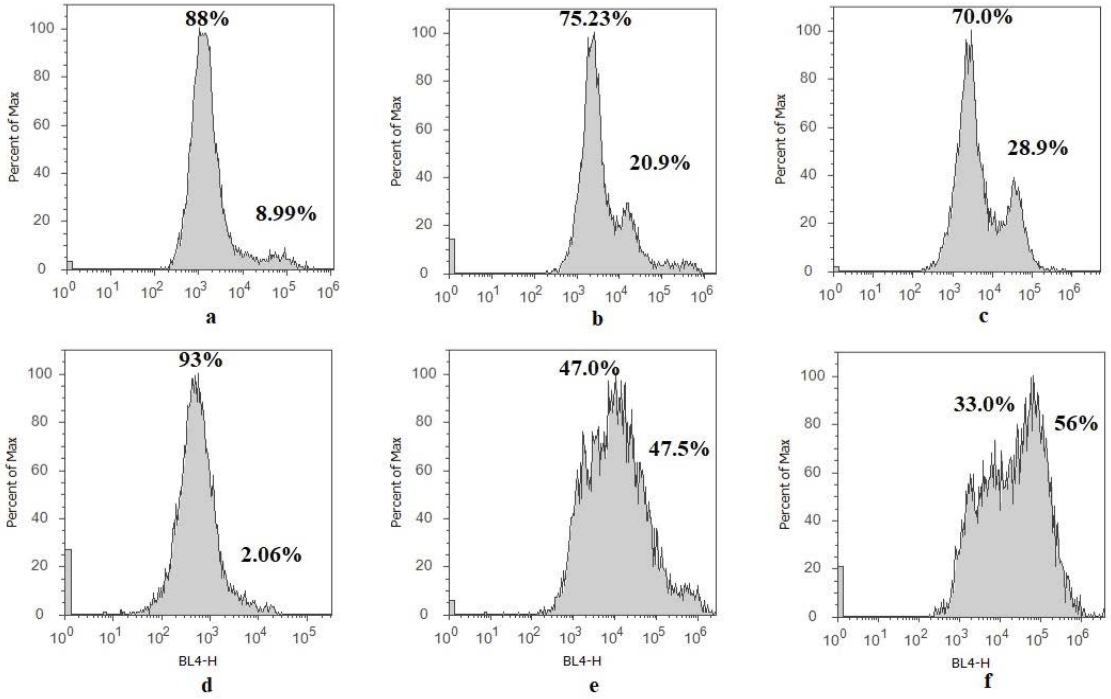

Figure 3 Cell cycle analysis in PLME treated T-47D and MDA-MB-453 cells. Cell cycle in T-47D and MDA-MB-453 cells was assessed after 24 h of treatment with PLME by PI staining and measured by flow cytometry analysis; Where; a): T-47D cells without drug; b): T-47D cells with PLME (20 µg/mL); c): T-47D cells with PLME (40 µg/mL); d): MDA-MB-453 cells without drug; e): MDA-MB-453 cells with PLME (20 µg/mL); f): MDA-MB-453 cells with PLME (40 µg/mL); Numbers indicate the percentage of cells in each gate /phase

Expression of Bak1 and Bax Family Proteins in PLME Treated Cells

The apoptosis-inducing potential of the extract was further tested in cancer cells using the Bcl2 family genes expression assay. The Bcl2 family members are pro-apoptotic proteins Bax, Bad, and Bak1 that promote cell death; while the anti-apoptotic proteins Bcl2 and Bcl-xL inhibit apoptosis.

In T-47D cells, PLME treatment resulted in a highly significant ($p<0.0001$ compared to untreated cells) down-regulation of Bcl2 (Figures 4a-4b). The increase in the concentration of PLME resulted in significantly ($p<0.05$,

p<0.001, p<0.0001 compared to untreated cells) increased expression of Bax and Bak1 (Figure 4c-4f). In MDA-MB-453 cells, PLME induces the significant upregulation (p<0.05, p<0.001, p<0.0001 compared to untreated cells) of BAK1 in 24 h (Figure 4d), whereas, BCL2 expression was downregulated (p<0.05, p<0.001, p<0.0001 compared to untreated cells) in PLME exposed cells (Figure 4b).

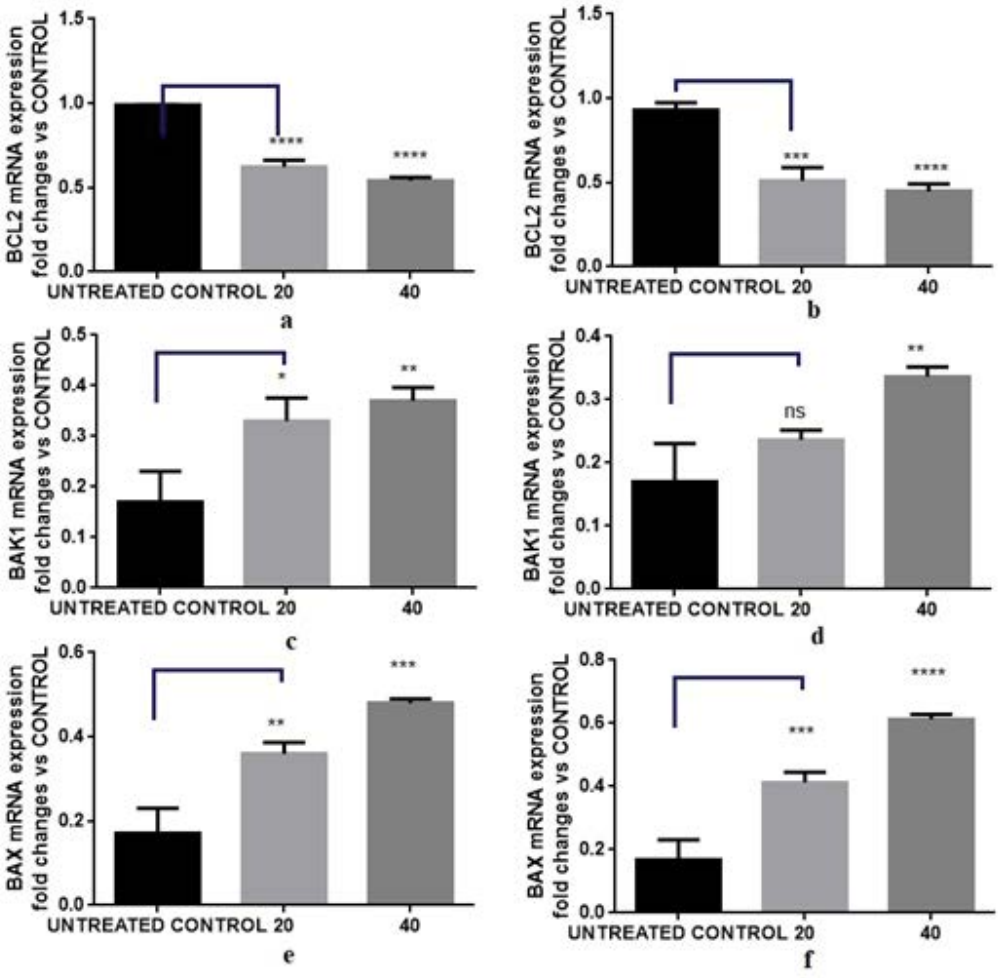

Figure 4 Regulation of Pro- and Anti-apoptotic gene expression in PLME treated T-47D and MDA-MB-453 cells; Here; a): Bcl2 mRNA expression in PLME treated T-47D cells; b): Bcl2 mRNA expression in PLME treated MDA-MB-453 cells; c): Bak1 mRNA expression in PLME treated T-47D cells; d): Bak1 mRNA expression in PLME treated MDA-MB-453 cells; e): Bax mRNA expression in PLME treated T-47D cells; and f): Bax mRNA expression in PLME treated MDA-MB-453 cells. The Bcl2, Bax, and Bak1 gene expressions were studied by using Real-Time PCR. The experiment was repeated three times and the above values of gene expression are the mean of three replicates in three different experiments. Data are expressed as the mRNA copies in cells, where significance refers to the differences between PLME treated and control untreated cells (n=3; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05, ns-not significant). Error bars indicate SD

DISCUSSION

The two breast cancer cells, MDA-MB-453 and T-47D were analysed for the evaluation of the anticancer mechanism of action of PLME. The T-47D is an invasive ductal carcinoma cell line, which possesses the ER+, PR+/-, and HER2- immunological profile [15,16]. The MDA-MB-453 breast cancer cell belongs to adenocarcinoma which is double-negative in ER and PR, HER2+ [17,18]. Apoptosis induction is a promising approach for cell population control in the treatment of cancer. In this study, the upregulation of Bak1, cell cycle arrest, and phosphatidylserine on the membrane

of T-47D and MDA-MB-453 cells confirmed the apoptosis in presence of PLME. Our finding suggests that PLME reduces the cell viability of both the breast carcinoma cells in both concentration and time-dependent manner. The induction of apoptosis by *P. longifolia* extracts proved its anti-proliferative action against KB and P-388 cells, HL60, MCF7, and Hela cells [4,7,19,20]. The previous report state that the induction of apoptosis is due to presence of the active components like flavonoids, tannins, saponins, steroids, and glycosides. A phytochemical study on the hexane extract of stem bark of PL has led to the characterization of various clerodane and ent-halimane diterpenes which are active constituents that are reported to have antitumor activity [21,22].

In the current study, we examined the morphological, biochemical, and molecular assays for the confirmation of apoptosis in PLME treated human breast cancer cells. This is represented as the first report of apoptosis in human breast carcinoma cells-T-47D and MDA-MB-453 cells. The anti-proliferative effect of PLME on T-47D cells and MDA-MB-453 cells was evaluated by MTS assay. The time and dose-dependent effect of PLME reflected promising remedies against Human breast carcinoma cells. The cell viability was reduced in the 24 h of the exposure to PLME. However, the prolonged exposure up to 48 h proves the positive effect of strong anticancer action of PLME. In previous research, *P. longifolia* extracts exhibited concentration-dependent cytotoxicity in Ehrlich's Ascites Carcinoma (EAC) and Dalton's Ascites Lymphoma (DLA) cells with IC50 values of 45.77 µg/ml and 52.52 µg/ml, respectively, whereas, IC50 values were reported for HeLa and MCF-7 cells were 25.24 µg/ml and 50.49 µg/ml, respectively [20]. Although the MTS assay proved that the PLME induced death in human breast cancer cells, the mode of cell death was further validated by Annexin V/PI and cell cycle analyses.

The Phosphatidylserine (PS) presence on the plasma membrane is a marker for mitochondria-mediated apoptosis in cells [23]. The Phosphatidylserine (PS) that appeared on the membrane of apoptotic cells have a high affinity to Annexin V [24]. In this study, Annexin V FITC/PI assay exhibited an increase in apoptosis when treated with *P. longifolia* compared to that of untreated T-47D and MDA-MB-453 cells.

In an exploration of the molecular mechanism in the induction of apoptosis, it is imperative to study the gene expression of anti-apoptotic and pro-apoptotic genes in PLME treated cells. BCL2 family proteins regulate the Mitochondria dependent apoptosis. The BCL2 binds to pro-apoptotic members such as Bax, preventing pore formation and cytochrome c release. BAX/BAK oligomerization leads to the release of several apoptogenic molecules from the mitochondrion [25]. This study reports the upregulation of BAK and BAX genes and downregulation of BCL2 genes in PLME treated T-47D and MDA-MB-453 cells. PLME induces the expression of proapoptotic genes in the cells and leads to activation of mitochondrial apoptosis pathway which might be mediated through the BAK and BAX proteins. Similar results were reported that *Polyalthia longifolia* induces the expression of BAX and BAK and inhibits the BCL2 expression [7].

The increased phosphorylation inactivates the BCL2 proteins when cells are arrested in the G2/M phase. This G2/M phase is controlled by a series of signalling cascades by which the cell replicates its DNA, divides, and proliferate. Anti-cancer drugs can activate p53-dependent and p53-independent pathways to arrest cancer cells at the G2/M checkpoint [26,27]. Verma, et al. reported the action of chloroform fraction on the enhanced annexin-V-FITC binding of the cells, increased sub-G0 DNA fraction, in HL-60 cells. In PLME treated T-47D and MDA-MB-453 cells, the G2/M phase cell population was found significantly increased than that compared with the untreated control cells [19]. This suggests that apoptotic cell death is due to the arrest of the cell cycle and the accumulation of cells in the G2/M phase.

The overview findings provide preliminary evidence of the efficacy of PLME against two different breast carcinoma cells. PLME arrest cell cycle at G2/M phase, upregulated pro-apoptotic gene expression, and downregulated BCL2 gene expression in invasive cancer cells-T47D and MDA-MB-453 cells. The research studies with further pharmacological investigations need validation to evaluate its activity and immune-modulatory effects in modified preclinical models of cancer for its translation as a potential anticancer agent.

CONCLUSION

The current investigations demonstrated the efficacy of methanolic extract of *Polyalthia longifolia* cv. *pendula* on T-47D and MDA-MB-453 cells. A significant insight towards the antiproliferative effect of PLME was validated by morphological, biochemical, and molecular modulations. The externalization of Phosphatidylserine on the membrane

of T-47D and MDA-MB-453 cells confirmed the effective induction of apoptosis. The cell cycle arrest at the G2/M phase and downregulation of BCL2, upregulation of BAX and BAK1 expression was observed in PLME treated cells. Thus, the present study postulates a rationale for the drug development of the PLME against Human breast cancer as a prospective of anticancer drugs mechanism.

DECLARATIONS

Authors' Contributions

Kiran Ramesh Kharat had performed the experimentation; Kiran Ramesh Kharat, Amol Kharat, contributed to the designing of the protocol and provided the help for the experimentation; Amol Kharat prepared the manuscript; Vinod R. Ragade participated in the discussion, protocol designing, editing, and revising the manuscript. All authors read and approved the final manuscript for submission.

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Availability of data and materials

Please contact the author for data requests.

Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Consent for Publication

This study has "Not applicable" to any person's data.

Ethics Approval and Consent to Participate

This study has "Not applicable" to any person's data.

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