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PIPERLONGUIMINE INDUCES CELL DEATH VIA ROS GENERATION, ATP DEPLETION, GLUTATHIONE DEPLETION AND DISSIPATION OF MITOCHONDRIAL MEMBRANE POTENTIAL IN HUMAN BREAST CANCER CELL LINE.

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ABSTRACT

Breast cancer is one of most common cancers that are associated with females. Reactive oxygen species (ROS) are formed during metabolic processes.ROS has dual role depending on their concentration in cells if concentration is low, ROS act as "redox messengers" in intracellular signalling but when ROS concentration is elevated it induce depletion of intracellular antioxidant such as glutathione (GSH).Consequence of ROS generation lead to superoxide anion production and ATP depletion.ROS generation cause mitochondrial membrane potential dissipation. Thus a natural product that induces ROS generation could be used as potential molecule for breast combating cancer. In present investigation we found that Piperlonguimine (PLG) a natural product inhibited breast cancer cell line MCF-7 by ROS generation. We investigated that ROS generation by PLG causes severe ATP depletion, dissipation of mitochondrial membrane potential and super oxide anion generation. Overall results demonstrated that antiproliferative effect of PLG was facilitated by ROS generation.

KEYWORDS: Piperlonguimine (PLG), Reactive oxygen species, ATP depletion, Glutathione depletion, Mitochondrial membrane potential

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INTRODUCTION

Breast cancer is common cause of death in females[1]. Even though taxol, vincristine, vinblastine, camptothecin are most widely used anticancer in breast cancer chemotherapy[2]. Finding of new natural product for anticancer therapy is never ending mission[3]. Apoptosis plays pivotal role cancer prevention[4]. Cancer is characterized by uncontrolled cancer cell growth, deregulation of cell cycle and metastasis[5]. A Hallmark feature of apoptosis is cancer cell death without inflammation. Apoptosis involves two types of pathways intrinsic and extrinsic pathway[6]. Mitochondria has crucial role in intrinsic pathway therefore it become prime target for cancer chemotherapy. Mitochondria causes release of pro apoptotic proteins that activate caspases which further lead to apoptosis induction. Mitochondrial membrane potential (MMP) is essential for cancer cell proliferation but dissipation in MMP leads to apoptosis induction [7]. Like a coin ROS has two sides, it acts as redox messengers at physiological low levels in intracellular signalling and regulation but during oxidative stress elevated level of ROS causes release of proapoptotic factors, loss of mitochondrial membrane potential, oxidative modification of DNA, lipid membrane and protein[8]. All these ROS induced oxidative modifications are implicated in apoptosis induction. Thus ROS generation could be one novel strategy for chemoprevention[9].ROS are having fascinating role as it helps for cell proliferation at the same time it promotes apoptosis induction[10]. During intrinsic apoptosis event, ROS generation causes oxidation of permeability transition pore protein brings significant increase in mitochondria anion flux. A significant increase in ROS causes dissipation of the mitochondrial membrane potential (Δψm),translocation of proapoptotic protein Bax and cytochrome c to cytosol[11].ROS production in mitochondria causes oxidation of mitochondrial DNA which encode 13 polypeptides of the respiratory chain. Oxidized mitochondrial DNA is an early event in mitochondria dysfunction.ROS generation causes oxidation of cardiolipin, oxidized cardiolipin detaches from cytochrome c and translocate to outer mitochondrial membrane and conjugated with truncated Bid which further form mega pore along with truncated Bid[12]. This mega pore is essential for cytochrome c translocation from mitochondria to cytosol. Elevated level of cytosolic cytochrome c leads to apoptosis induction. Elevated level of ROS causes intracellular depletion of GSH which brings apoptosis induction[13]. Plant derived natural products contain bioactive compounds with antiproliferative activities. These natural products can be used as alternative therapeutic or preventive regimens for individuals with cancer. Piperlonguimine (PLG) is a natural alkaloid of the long pepper (Piper longum) that can selectively kill cancer cells[14]. It is well documented that PLG showed anti-platelet aggregation, anti-inflammatory and anticancer properties[15].PLG exerts anti cancer effect by activating p53 expression.PLG exhibited anti cancer effect by generating ROS in cancer cells[16]. The objective of present study was to delineate apoptosis induction in breast cancer cell line (MCF-7). In current study we accessed antiproliferative role of PLG in MCF-7. Our experimental results clearly showed PLG inhibited MCF-7 in dose dependent manner.PLG caused severe ATP depletion in MCF-7.ROS played crucial role in apoptosis induction in MCF-7 cells.PLG induced ROS generation leads to loss of MMP, GSH depletion and superoxide production.

MATERIALS AND METHODS

Piperlonguimine purchased from Biovison USA,LDH assay kit purchased from Cayman Chemical, USA, MTT, DMSO, Nitro blue tetrazolium,JC-1 (5,5',6,6' tetrachloro-1,1',3,3' - tetraethylbenzimidazolocarbocyanine iodide) purchased from sigma. EnzyLight™ ADP/ATP Ratio Assay Kit from BioAssay Systems, USA

Cell Culture and Maintenance

MCF-7 breast cancer cell line obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were grown in monolayer

culture in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) containing 10% fetal bovine serum (FBS; Sigma–Aldrich) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37^0C. For all experiments DMEM containing 10% FBS was used.

1. MTT Assay

Briefly, exponentially growing MCF-7cells were plated in 96-well plates (1×10^4cells/well) and cultured for 24h. Then, cells were incubated with increasing concentrations of PLG in DMEM supplemented with 5 % FBS. After 48h of treatment, 20 µl MTT solution (2.5 mg/ml) was added to each well, followed by incubation for 4 h at 37^0C with 5% CO_2. The MTT solution was removed, and 200 µl DMSO was added to each well to dissolve the blue MTT formazan crystal that had formed. Absorbance was measured at 550 nm using a microplate reader.

2. LDH assay

Cell membrane integrity was evaluated using LDH assay.LDH Enzyme activity was expressed as the percentage of extra cellular LDH activity of the total LDH activity of the cells. LDH assay was carried out using the commercially available kit for in vitro cytotoxicity evaluation (LDH Assay Kit, Cayman chemical USA). Briefly, exponentially growing MCF-7cells were incubated with different concentrations of PLG for 48 h , centrifuged at 300×g for 5 min. Then supernatant of each well was collected to a fresh flat bottom 96-well culture plate and further processed for enzymatic analysis as per the instructions given in the kit.

4. Measurement of ADP/ATP ratio

The ADP/ATP ratio as an indicator of cell proliferation, necrosis, and apoptosis was measured using EnzyLight™ ADP/ATP Ratio Assay Kit from Bioassay Systems, USA, according to instructions provided by the supplier. Briefly, 1×10^4 cells/well were treated with different concentrations of PLG, taken in 96 well white opaque microplate. At the time of assay, remove the culture medium immediately from control and treated MCF-7cancer cells before adding 90 µL ATP Reagent. Add 90 µL ATP Reagent to each well and mix by tapping the plate. After 1 min, read luminescence (RLU A) on a luminometer. Ten minutes after reading the luminescence for ATP (RLU A), read the luminescence of the samples again (RLU B). This measurement provides the background prior to measuring ADP (i.e. the residual ATP signal).Immediately following reading RLU B, add 5 µL ADP Reagent to each well and mix by tapping the plate or pipetting up and down. After 1 min. read luminescence (RLU C) on a luminometer.

Calculation of ADP/ATP Ratio =RLU C – RLU B/ RLU A

5. Mitochondrial membrane potential determination using JC-1 dye

Mitochondrial membrane potential changes were measured with JC-1 dye as described in[17].JC-1 in aggregated form emits a red light, and in the monomer form emits green light.MCF-7 cells were seeded to a 24 well plate, in plain medium and treated different concentrations of PLG. After incubation for 48h, the medium was discared and plain medium containing JC-1 dye was added to each well. The cells were incubated at 37^0C in 5% CO_2 incubator for 15 min, and then the staining solution was discared. The cells were washed once with phosphate buffered saline and re-suspended in phosphate buffered saline. The fluorescence was measured in a Biotek spectrofluorometer. The measurement for red fluorescence was taken at Ex—550 nm and Em—590 nm and for green fluorescence was measured at Ex—490 nm and Em—535 nm. The mitochondrial membrane potential status is expressed as red fluorescence to green fluorescence ratio.

6. Intracellular ROS determination using DCFDA

The intracellular ROS was measured by fluorescent probe, 2,7-dichlorofluorescein diacetate (DCFH-DA) as described in[17]. MCF-7 cells are incubated with different concentrations of PLG and co-treated with DCFH-DA at final concentration 5 µM and incubated at 37^0C up to

3 h. Read fluorescence intensity at Excitation—485 nm and Emmssion—530 nm.Amount of ROS is proportional to DCF intensity.

7. Glutathione determination

Intracellular GSH determination was carried out using monobromobimane (MBB) as described in [18]. Briefly, exponentially growing MCF-7cells were plated in 96-well plates (1×10^4cells/well) and incubated with different concentration of PLG for 48 h then treatment media was removed and then add media containing 8 µM monobromobimane (MBB). Read fluoroscence at excitation 360 nm and emission at 460 nm.

8. Measurement of superoxide anion production

Superoxide anion production was measured using a colorimetric NBT assay as described in[19],where in superoxide anions convert water soluable NBT to formazan crystal.

Exponentially growing MCF-7cells were plated 1×10^4 cells/well in 96-well flat-bottom tissue culture plates and treated for 48 h with different concentrations of PLG. Each well also received 0.02 ml of 2% NBT in medium at initiation of culture. After 6 h culture supernatants were removed and formazan crystals were dissolved in 0.2 ml DMSO. Absorbance was measured at 570 nm using Biotek spectrofluorometer.

9. Inhibitory study

In order to define the role ROS, cell viability of breast cancer cells was measured using MTT assay.MCF-7 cells are incubated with PLG and N-acetyl cysteine which acts as antioxidant.

10. Statistical analysis

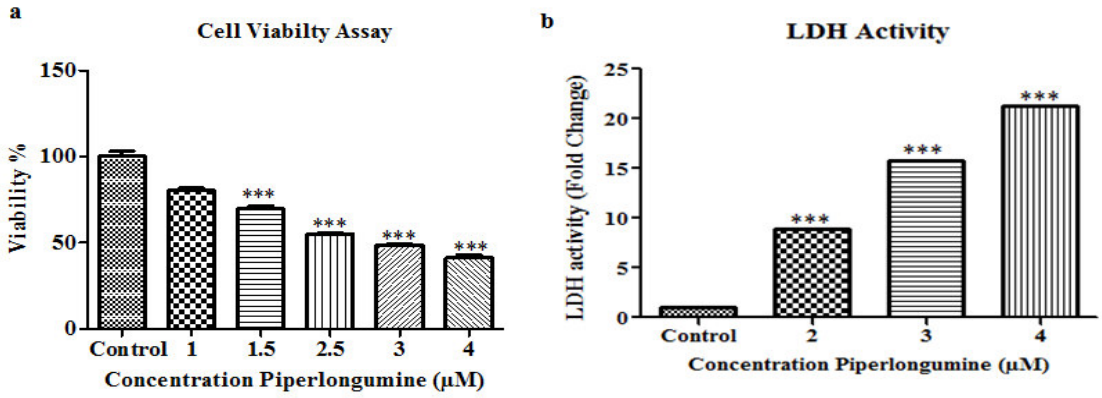
The data are expressed as mean ± SE from triplicate replicates per treatment. Data were analyzed by one-way ANOVA followed by Dunnet's test for comparison between control and treatment groups. The level of significance was set at P≤ 0.05. Data of all the results in this study were obtained from at least three independent experiments with a similar pattern.

RESULTS

1. PLG inhibits MCF-7 in dose dependent manner

By MTT assay, we first accessed the effects of PLG on the growth of human breast cancer cell line. PLG significantly inhibited the proliferation of cell lines in a dose-dependent manner .On the other hand, viability of normal cells was minimally affected following treatment to high concentrations of PLG that were highly cytotoxic to cancer cells. To determine plasma membrane integrity LDH assay was carried out. Exposure of MCF-7 cell to different concentration of PLG for 48h resulted in significant dose dependent increase in LDH release at 2µM,3µM and4µM LDH release fold change was around 9,16 and 21respectively whereas control cells showed decrease in LDH release.

Figure 1



a. MCF-7 cells were incubated with1µM, 1.5µM, 2.5µM, 3µM and 4µM concentrations of PLG for 48h. MTT Assay was performed and cell viability was determined. ***p<0.001 Vs control (one-way ANOVA).Data represents the mean ± SEM of 3 independent experiments. **b.** MCF-7 cells were incubated with 2µM, 3µM and 4µM concentrations of PLG for 48h. LDH Assay was performed and LDH activity was determined. ***p<0.001 Vs control (one-way ANOVA).Data represents the mean ± SEM of 3 independent experiments.

2. PLG induced ATP depletion

The changes in ADP/ATP ratio have been used to determine apoptosis. Increased levels of ATP and decreased levels of ADP have been associated with cancer cells, whereas decreased levels of ATP and increased levels of ADP are recognized in apoptotic cells. To elucidate cytotoxicity of PLG in MCF-7 was because of ATP depletion, MCF-7 cells were treated with different concentrations of PLG for 48h and ADP/ATP ratio was determined using bioluminescent measurement of ATP. PLG treated MCF-7 cells showed significant increase in ADP/ATP ratio in dose dependant manner at 3µM and 4µM ADP/ATP was 0.26 and 0.63 respectively, whereas control cells showed decline in ADP/ATP ratio. This finding implies that ATP depletion played crucial role in PLG induced apoptosis in MCF-7 cells

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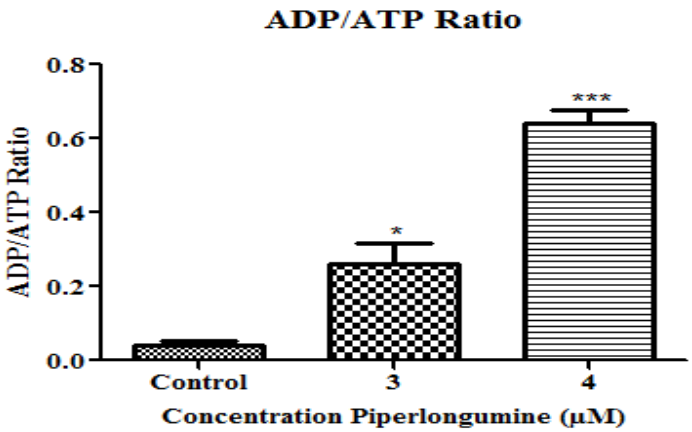
Fig.2.

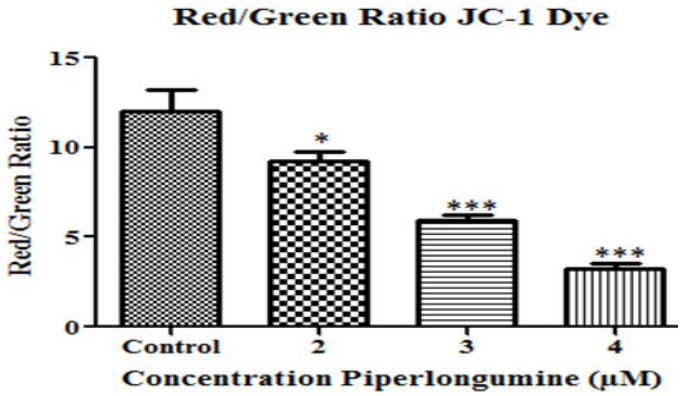


MCF-7 cells were treated with 2µM, 3µM and 4µM concentrations of Piperlonguimine for 48hrs and ADP/ATP ratio was determined using a bioluminescent kit as described in 'Materials and Methods'. *p< 0.05, ***p<0.001 Vs control (one-way ANOVA).Data represents the mean ± SEM of 3 independent experiments.

3. PLG triggers mitochondrial membrane potential (ΔΨm) dissipation

Dissipation of MMP is one of crucial biomarker for apoptosis as it facilitates release of cytochrome c and activation of proapoptotic proteins that leads to mitochondria mediated apoptosis. In order to delineate mitochondrial mediated apoptosis, MCF-7 cells are treated with different concentrations of PLG for 48h and MMP was determined using MMP sensitive JC1 dye which forms red aggregates in healthy cells because of polarized mitochondria and forms green monomers in apoptotic cells as of depolarized MMP. Red/Green ratio signify status of MMP and determined by spectrofluorometric analysis.PLG treated MCF-7 cells exhibited decrease in Red/Green ratio in dose dependant manner at 2µM,3µM and4µM Red/Green ratio was 9.23,5.97,3.23 respectively whereas control cells showed increase in Red/Green ratio. Collectively, these results indicated that PLG-mediated inhibition of MCF-7 cells proliferation was due to dissipation of MMP.

Fig.3.

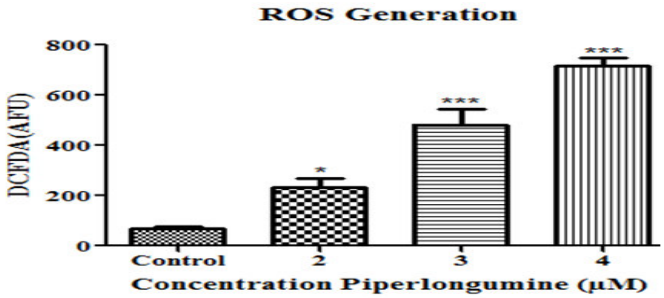


MCF-7 cells were treated with 2µM, 3µM and 4µM concentrations of Piperlonguimine for 48hrs and Red/Green ratio was determined by using JC-1 dye. *p< 0.05, ***p<0.001 Vs control (one-way ANOVA).Data represents the mean ± SEM of 3 independent experiments.

4. PLG induced ROS generation

Elevated levels of ROS have been suggested as the mechanism of action of various antiproliferative agents in cancer cells. To evaluate ROS generation in apoptosis events Fluorimetric analysis was performed using DCFDA which is ROS sensitive dye[17]. In this study MCF-7 cells were treated with different concentration of PLG for 48 h. Results illustrated that PLG treated MCF-7 cells showed significant elevation in ROS generation. Taken together results implied that ROS generation played important role PLG induced apoptosis in MCF-7 cells.

Fig.4.

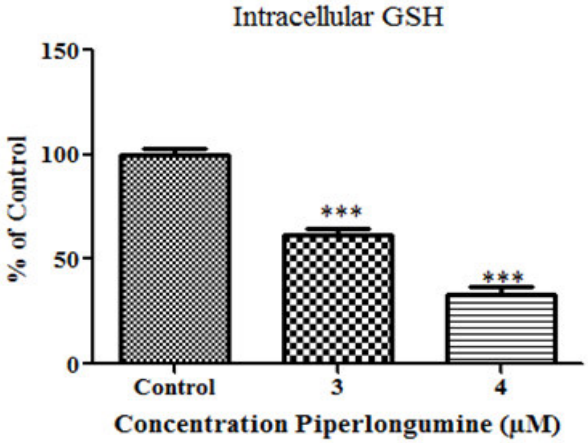


MCF-7 cells were treated with 2µM, 3µM and 4µM concentrations of PLG for 48hrs and ROS was determined by using DCFDA dye. *p<0.05, ***p<0.001 Vs control (one-way ANOVA).Data represents the mean ± SEM of 3 independent experiments.

5. GSH depletion by PLG

ROS generation is characterized by decline activity of antioxidant enzymes such GSH, SOD, catalase. To validate the role of GSH depletion, Fluorimetric assay was carried out using monobromo bimane which detects intracellular GSH level. In the present study MCF-7 cells were incubated with different concentration of PLG for 48h.These results explained that GSH level was decline significantly in concentration dependant manner at 3µM and 4µM GSH level was 61.43% and 33.13% respectively. These obeservations suggested that GSH depletion was one of the major players for PLG induced apoptosis in MCF-7 cells.

Fig.5.

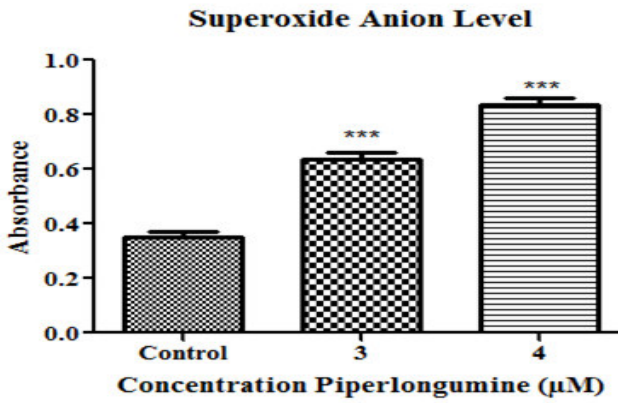


MCF-7 cells were treated with 3µM and 4µM concentrations of Piperlonguimine for 48hrs and Intracellular GSH depletion was determined by using monobromo bimane. ***p<0.001 Vs control (one-way ANOVA).Data represents the mean ± SEM of 3 independent experiments.

6. Superoxide anion production by PLG

Oxidative stress induced production of free radicals includes superoxide anion which has tendency to cellular DNA and induce apoptosis. To know the role of superoxide anion in apoptosis NBT assay was carried out. In this present study MCF-7 cells were treated with different concentrations of PLG for 48h. Results showed that superoxide anion production increased in concentration dependant manner in PLG treated MCF7 cells. Overall results authenticated that antiproliferative effect of PLG in MCF-7 cells was due to oxidative stress induced formation of superoxide anion that activate cell death.

Fig.6.



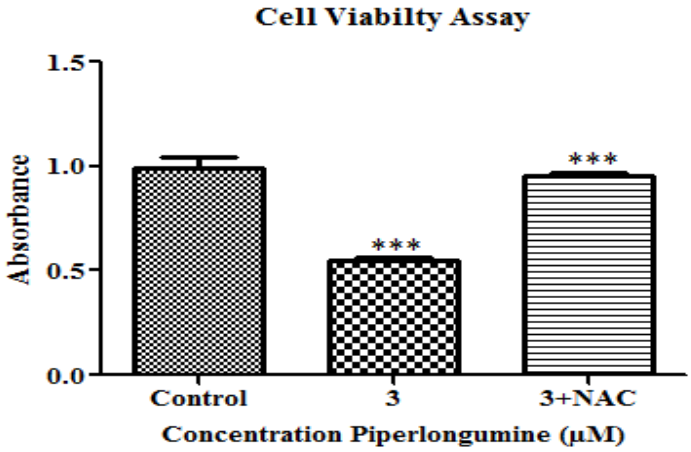
MCF-7 cells were treated with 3µM and 4µM concentrations of PLG for 48hrs and superoxide anion level was determined by using Nitro blue tetrazolium . ** p<0.01,***p<0.001 Vs control (one-way ANOVA).Data represents the mean ± SEM of 3 independent experiments.

7. Inhibitory Study

To unveil the role ROS in cancer cell viability we carried out MTT assay for cell viability in which MCF-7 cell were incubated with PLG and n-acetyl cysteine which acts as antioxidant. Results revealed that PLG caused significant decline in cell viability whereas ROS generated

by PLG were scavenged by N-acetyl cysteine and retained cancer cell viability. This report confirmed that PLG induced cell death was because of ROS generation.

Fig.7.



MCF-7 cells were incubated with 3µM and N-acetyl cysteine concentrations of PLG. MTT Assay was performed and cell viability was determined. ***p<0.001 Vs control (one-way ANOVA) Bonferroni's Multiple Comparison Test. Data represents the mean ± SEM of 3 independent experiments.

DISCUSSION

Cancer is uncontrolled cell growth phenomenon[20]. Cell viability determination is one of the most vital parameter during evaluation of antiproliferative properties of anticancer drug. Results revealed that MCF-7 breast cancer cell line was PLG sensitive cell line. Cell death is characterized by cell membrane damage that instigates LDH release. In present study PLG exhibited significant elevation in LDH release which has given clear impression that PLG induced cell death was facilitated by damage to cell membrane integrity. Proliferation of cancer cells requires ATP for their survival[21]. Decrease in ATP production leads to apoptosis[22]. The role of ATP during apoptosis was evaluated using ATP bioluminescent assay [23].Our study results showed PLG induced a significant augmentation in ATP depletion in MCF-7cells.From this result we concluded that ATP depletion was responsible for PLG induced apoptosis in MCF 7 cells. Mitochondrial membrane potential is crucial for proliferation of cancer cell. Change in MMP leads to pore formation in mitochondria which in turn activates proapoptotic proteins that promotes release of cytochrome c and thus induces intrinsic apoptosis pathway[24]. Present study explored MMP significance in apoptosis. Decline Red/green ratio in PLG treated cell explicates MMP dissipation in MCF-7 cells. Thus our study unveiled dissipation of MMP was essential for apoptosis induction by PLG in MCF-7 cells. Oxidative stress plays pivotal role in ROS generation which leads to apoptosis induction.ROS generation causes mitochondrial dysfunction that initiates cytochrome c release which further activate intrinsic apoptosis pathway[25]. Thus ROS generation is instrumental for apoptosis induction. In present study evaluation of ROS was carried out using DCFDA dye and results demonstrated that after PLG treatment ROS generation increased in MCF-7 cells.ROS generation has direct effect on antioxidant enzymes such as GSH and SOD. Depletion of these enzymes due to ROS generation make cancer cell prone to apoptosis. The role of antioxidant enzymes was evaluated using monobromobimane. Results demonstrated that PLG induced elevated level

of ROS causes depletion of GSH. Superoxide anion (SOA) is free radical that damage protein, DNA and plasma membrane by oxidation. In present study the role SOA was appraised by NBT assay and results indicated that SOA level increased in PLG treated MCF-7cancer cells. Cell viability assay in presence of N-acetyl cysteine showed that ROS generation by PLG causes significant cell death but in presence of antioxidant cell viability was increased. This expound that PLG induces cell death via ROS generation. Overall observations divulged imperative role of ROS generation in PLG induced apoptosis in MCF 7 cancer cell line.

CONCLUSION

PLG inhibited MCF-7 cell growth in concentration dependent manner. Apoptosis induction by PLG was due to ROS generation that caused ATP depletion and loss of MMP. More over ROS generation leads to severe depletion of antioxidant such as glutathione, which made cancer cells prone to apoptotic cell death. Over all data interpreted anticancer potential of PLG in breast cancer cell line.

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