Cancer Research and Medicine
DOI: https://doi.org/10.63626/bq2axz17
ISSN Online: 3104-4131
ISSN Print: XXXX-XXXX

Research Article

Erufosine mediated regulation of apoptotic, cell cycle and cell survival genes in breast cancer cells

Ali Raza Abdullah[1], Talha Saleem[2]
1. Health and Population department Tehsil Headquarter Hospital Arifwala, Pakistan
2. Department of Medical Laboratory Technology, Superior University, Lahore, Pakistan

Abstract
Background: Breast cancer is a common cancer in females worldwide with continuously growing incidence over the decades. Novel compounds are needed to treat breast cancer effectively. Erufosine, a synthetic lipid, is a promising anticancer agent that acts on cell membranes to induce antitumor effects and interfere with lipid homeostasis leading to toxic effects.
Methods: Toxic effects of erufosine on human breast cancer cell lines were determined by MTT dye assay. Cell lines (MCF-7 and MDA-MB-231) were cultured and treated with erufosine for 24-72 h. Expressional changes in apoptosis (BCL2), cell cycle (CDKN1A) and cell survival (MYB) related genes were determined by real-time PCRs.
Results: The compound induced cytotoxic effects in the cells in concentration dependent format. Both cancer cell lines were found sensitive towards the exposure with erufosine. Selected genes were de-regulated by the compound in cell specific format. Cell cycle inhibitor gene (CDKN1A) was up-regulated while cell survival (MYB) was down-regulated in the cells. Apoptosis (BCL2) gene showed a variable response in the two cell lines.
Conclusion: Erufosine induces cell death in breast cancer cells and de-regulate the associated genes. Erufosine mediated observed functional and molecular changes in breast cancer cells highlight its antineoplastic potential.
Key Words: Erufosine, Apoptosis, Cell cycle, Breast cancer, Anticancer

INTRODUCTION

Alkyl-phospholipids are the synthetic molecules having similarity with endogenous lipids of cell membrane. There are different generations of alkyl-phospholipids synthesized over the last decades with structural modifications for improved biological and therapeutic efficacy against different diseased conditions. One basic alkyl-phospholipid changed by the substitution of the choline bunch by a heterocyclic piperidine gathering that improved the remedial power alludes, this also made it a steady compound with a half-life of around 140 h named perifosine (octadecyl-1-(1,1-dimethyl-piperidinio4-yl)- phosphate). Perifosine was having a significant bit of leeway of strength over past alkyl-phospholipid [1].

Corresponding Author: Talha Saleem
Email: talha.saleem@superior.edu.pk
ORCID: 0000-0002-0631-1286
Received: 23.06.2025
Revised: 26.07.2025
Accepted: 26.07.2025
Published: 27.07.2025

Two additional analogs of alkyl-phospholipids, erucylphosphocholine (ErPC) and erufosine (ErPC3) were incorporated, having a more drawn out 22 carbon chain with a ω-9-cis-twofold security. This alteration empowers intravenous organization because of the decreased hemolytic action than previously alkyl-phospholipid [2]. These alkyl-phospholipids are moderately less hemolytic on the grounds as they exist as lamellar structures in fluid arrangements rather than micelles like different other alkyl-phospholipids [3]. Another intriguing element of these new analogs alludes to their capacity to cross the blood-cerebrum hindrance. Human glioblastoma cell lines experienced cytotoxicity [4].
Erufosine, a third era compound of alkyl-phospholipids demonstrated antineoplastic specialist for cell lines representing leukemia, numerous myelomas, prostate, breast and squamous malignant growth. Erufosine as other alkyl-phospholipids, has resemblance with endogenous phospholipids, intercepted lipid homeostasis that caused cell stress and apoptotic death in different malignant growth cell lines. Erufosine forced anticancer impacts in different cell lines by apoptosis, autophagy and G2 cell cycle [5].

Erufosine also intercepted cholesterol homeostasis, generations of ATPs and proteins related to cytoskeleton, these change lead towards stress, absence of vitality and migratory activities respectively. Erufosine is metabolically stable with decreased harmfulness towards GI tract and less hemolytic action *in vivo*. These qualities empowered the intravenous implementation of erufosine to accomplished pertinent clinical fixations, which was impractical with other alkyl-phospholipids [6].

The qualities of erufosine separating it from past alkyl-phospholipids include the structural change that made it less hemolytic than previous generation subsequently making its intravenous application achievable [7]. Erufosine can cross blood-cerebrum boundary along these lines which empowered it for the treatment of brain tumors. An *in vitro* examination uncovered glioblastoma cells sensitive to erufosine [4]. Erufosine comparatively is less lethal for bone marrow than other alkyl-phospholipids authorizing its higher intravenous dosages as monotherapy and mix regimens [8].

Pre-clinical investigations have uncovered the substantial potential of this compound as anticancer entity against breast cancer. Dineva and colleagues investigated erufosine for its antiproliferative movements both *in vitro* and *in vivo*. Two cell lines MDA-MB-231 and MCF-7 were utilized for *in vitro* examination and were found with similar sensitive to erufosine at IC50 of 40µM. An *in vivo* examination on instigated carcinoma in rodents uncovered over 85% reduction of tumor with just 7% body weight reduction. It is additionally presumed that erufosine impacts PI3K/AKT and Ras/Raf/MAPK pathways by decline in phosphorylation [9].

Anticancer potential of erufosine is also established against other cancers in laboratory settings. Erufosine was found to promote apoptosis in interminable lymphocytic leukemia at IC50 of 22µM [10]. It is additionally uncovered that retinoblastoma dependent signaling pathway is vital for antineoplastic action of erufosine [11]. BV-173 and K-562 cell lines were seen sensitive towards erufosine [12]. Erufosine in combination with antisense oligonucleotide synergistically restrain development of CML cells with no influence on development of normal cells [13]. Human and rodent colorectal cancer cell lines have been investigated as affectable by erufosine. Human cell line, SW480 was seen sensitive to erufosine at IC50 of 3.4 µM and rodent cell line CC531 was seen as sensitive to erufosine at IC50 of 25.4µM [6]. Human prostate malignancy cell lines DU-145, LNCaP and PC3 have been affected by erufosine [14]. Erufosine was also found to cause apoptosis, autophagy and the capture of G2 cell cycle in oral squamous carcinoma cell lines (HN-5 and FaDu) [15].

METHODS

Erufosine and Cell Lines

Source material, erufosine, was dissolved in PBS and stored in freezing conditions. Human breast cancer cells (MDA-MB-231 and MCF-7) were cultured in standard cell culture conditions while using RPMI-1640 medium along with 2mM L-glutamate, 10% fetal bovine serum and streptomycin/ penicillin (100µg/ml/100IU/ml).

Toxicity Assessment Assay

After treatment with erufosine for 24-72h, MTT assay was performed to monitor cell viability and proliferation. For this purpose, cell lines were seeded in culture plates and then treated with different concentrations of erufosine (0.75-100µM). Afterwards, MTT solution (5mg/ml) was added and incubated at standard conditions for 4h. Formation of formazan crystal by viable cells was dissolved to perform a colorimetric assessment. For this purpose, 100µL of the acidic solvent (0.04 N HCL/ 2-propanol) was added in each well and optical density was measured with the help of an ELISA reader (545nm) and numerical values were converted into percentage survival while comparing the results with untreated cells as controls.

Expression Analysis

Treatment with Erufosine

The cancer cell lines (MDA-MB-231 and MCF-7) were cultured (150,000cells/well/2ml medium) in 6-well culture plates and treated with three inhibitory concentrations (IC) of erufosine (IC_{25}, IC_{50}, IC_{75} determined from toxicity assay) for 48h. After treatment intervals, the cell palettes were collected for RNA extraction.

RNA Extraction

Total RNA was extracted from the control and treated cell palettes by using RNeasy Mini Kit (Qiagen), while following the manufacture's protocol. Briefly, the cell pellets were washed with PBS and a total of 600µl lysis buffer was added per sample and mixed well by pipetting to lyse the cells. A total of 360µl ethanol (96-100%) was added per sample and mixed by pipetting to precipitate RNA. Afterwards, the lysate was transferred to the spin column and centrifuged. After centrifugation the RNA present in the column was washed with provided Buffer 1 (700µl) and Buffer 2 (600µl) as recommended. At the end, 60µl nuclease free water was added to the column to elute RNA by centrifugation and stored at -80°C immediately for further use.

cDNA Synthesis and Verification

RNA was quantified with help from the spectrophotometric system, Nanodrop ND2000. A total of 1000ng extracted total RNA per sample was used to synthesize cDNA (40µl) by using Maxima Reverse Transcriptase (Thermo Scientific). Synthesized cDNA samples were verified with the help of PCR that is based on amplification of a reference gene (GAPDH). The amplification products were visualized by agarose gel electrophoresis method.

Primer Designing and Optimization

Primers for the three selected genes, i.e., BCL2, CDKN1A and MYB were designed by choosing gene sequence of selected genes from NCBI Gene bank and using Primer3 software. Primer sequences are given in Table 1 and were optimized by using gradient PCR method, while applying various Tm temperatures as per suggested by the manufacturer. The amplified products were visualized via agarose gel electrophoresis.

Real Time PCRs

qRT-PCR was performed for the selected 3 genes (as mentioned above) by using cDNA samples from cell lines (MDA-MB- 231 and MCF-7) treated with three concentrations of erufosine (IC_{25}, IC_{50} or IC_{75}) for 48h. SybrGreen florescence dye along with optimized primers were used for this purpose. $2^{-\Delta\Delta CT}$ method was used to find expressional changes in three selected genes in control and erufosine treated samples. β-actin was used as a reference gene in these experiments. Samples from untreated samples were used as control to find the fold changes in response to erufosine exposure.

Table 1: Primer Sequences for cDNA Check an qRT PCR of selected genes

Genes	Primer Sequence (F.)	Primer Sequence (R.)	Size (bp)
BCL2	GCCCTGTGGATGACTGAGTA	CGTACAGTTCCACAAAGGCA	(86)
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTC	(260)
MYB	CCCTGAGAAGGAAAAGCGAA	TGGTTCTGTGTTGGTAGCAC	(96)

RESULTS

Toxic Effects of Erufosine in Breast Cancer Cells

Erufosine toxic effects against breast cancer cells were studied by MTT dye reduction assay. Human breast cancer cell lines (MDA-MB-231 and MCF-7) cultured in RPMI-1640 medium were treated with different concentrations of erufosine. ICs of the test compound were determined via using GraphPad Prism 6 software. Inhibitory effects were calculated numerically as percentages of untreated controls. The growth inhibition was noticed after exposure of the cells with erufosine for three different time points with maximum effects in later time intervals that is 72h with highest (100µM) erufosine concentration. Overall, a significant decline was also observed in cells survival rate when exposed to various concentrations of erufosine for 24, 48 and 72h as shown in Figure 1. With the help of GraphPad Prism 6 software ICs were identified and respective IC_{50} values of erufosine against the breast cancer cell lines (MDA-MB-231 and MCF-7) are given in Table 2. Overall, both breast cancer cell lines were found sensitive towards the erufosine exposure.

Table 2: IC_{50} concentrations after 24, 48 and 72h of treatment with erufosine

	Breast Cancer Cells	
	MDA-MB-231	**MCF-7**
24 h	8µM	8.2µM
48 h	12.5µM	17µM
72 h	20µM	40µM

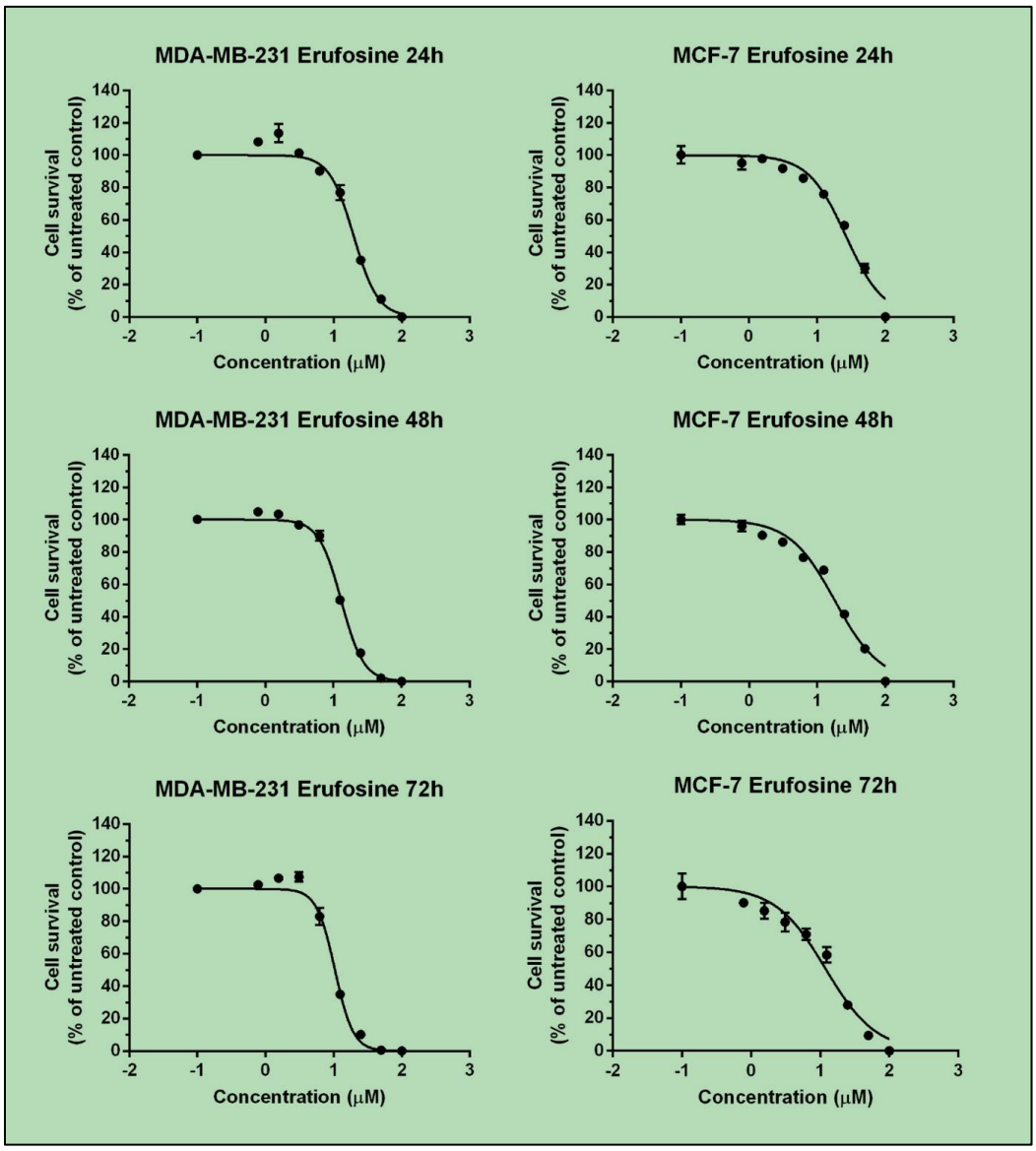

Figure 1: Cell lines were treated with erufosine, and cytotoxic effects were calculated by MTT assay. Numerical percentages compared to untreated controls were considered. Growth curves were generated by using GraphPad Prism software.

Real Time PCR and Expression Analysis

The breast cancer cell lines (MDA-MB-231 and MCF7) were treated with erufosine [low (IC_{25}), medium (IC_{50}) and high (IC_{75}) concentrations] followed by RNA extraction, cDNA synthesis and verification via amplification of a reference gene (GAPDH). Agarose gel electrophoresis methodology was used to visualize the amplified product of samples (Figure 2). Three genes (BCL2, CDKN1A, MYB) were selected for the expression analysis. Primers were designed and optimized by gradient PCR methodology using three different annealing temperatures (56°C, 59°C and 62°C). The amplified products showed specific amplification of the selected genes at all checked temperatures as shown in figure 3A-C. After primer optimizations, the three selected genes were amplified via real-time PCRs to validate the panel assay results. Effects of erufosine on the above-mentioned three genes selected were evaluated in breast cancer cell lines after exposure with three different concentrations of the compound. The results identified the concentrations dependent effects of erufosine on the selected genes in the cell lines. Cell cycle inhibitor gene (CDKN1A) was induced in the two cell lines after erufosine exposure; however, this induction was more prominent in triple negative breast cancer cells (MDA-MB-231). In converse, cell survival oncogene (MYB) was inhibited in the cells, where more intense downregulation was witnessed in receptor positive breast cancer cells (MCF-7). Pro-apoptotic gene (BCL2) showed variable response, where it was induced in MDA-Mb-231 cells especially at low concentrations of erufosine, while in MCF-7 cells, it was continuously inhibited after the treatment (Figure 4, 5).

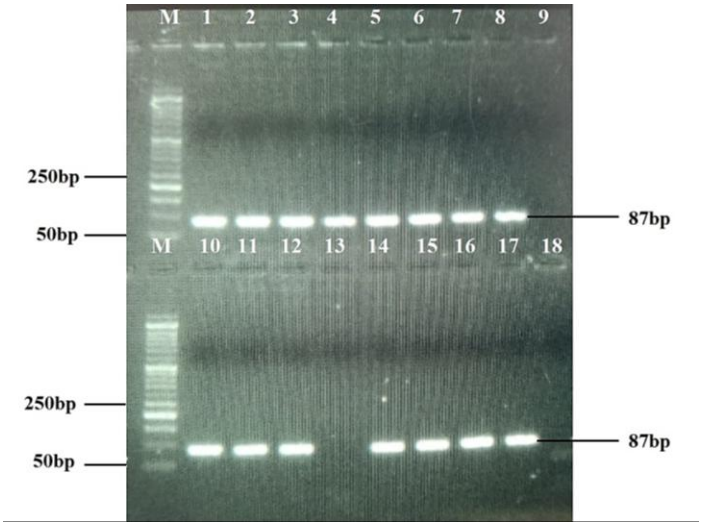

Figure 2: Electrophoresis of amplified cDNA of GAPDH gene the cells for cDNA verification. Sample distribution: Well M: DNA marker (50bp) Well 1-2: MDA-MB-231 untreated control, Well 3-8: MDA-MB-231 treated with erufosine, Well 9: Negative control, Well 10-11: MCF-7 untreated control, Well 12-17: MCF-7 treated with erufosine, Well 18: Negative control.

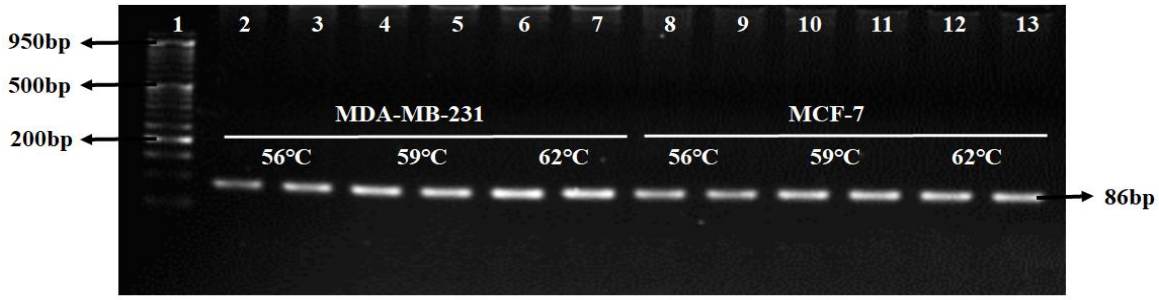

Figure 3A: Primers for BCL2 gene showed specific amplification at all tested annealing temperatures. Sample distribution:, Well 1: DNA marker (50bp), well 2-7: MDA-MB-231 cell line, and well 8-13: MCF-7 cell line.

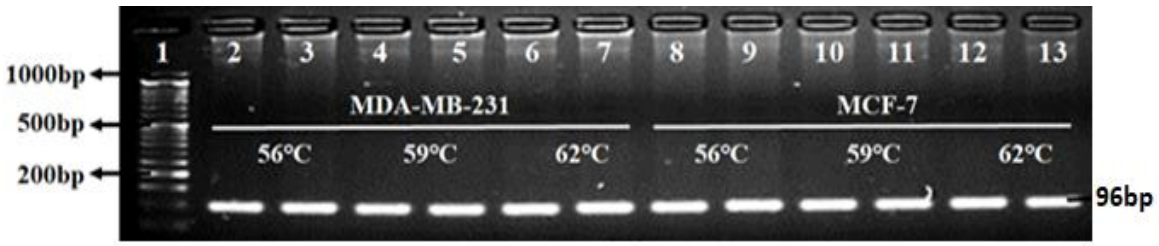

Figure 3B: Primers for MYB gene showed specific amplification at all tested annealing temperatures. Sample distribution: well 1: DNA marker (50bp), well 2-7: MDA-MB-231 cell line, and well 8-13: MCF-7 cell line.

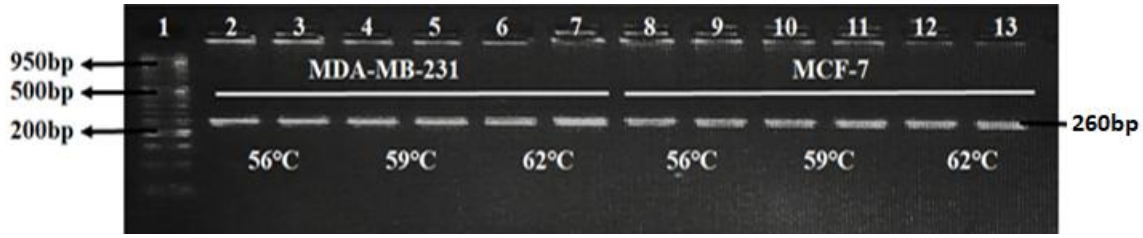

Figure 3C: Primers for CDKN1A gene showed specific amplification at all tested annealing temperatures. Sample distribution: Well 1: DNA marker (50bp), well 2-7: MDA-MB-231 cell line, and well 8-13: MCF-7 cell line.

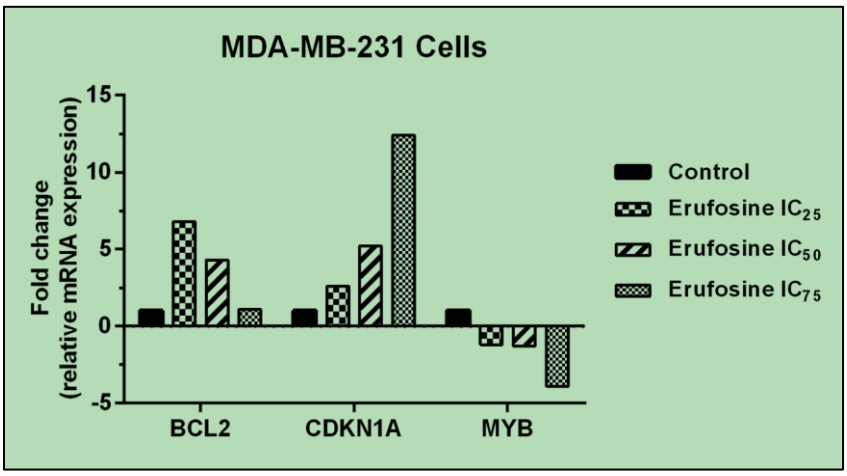

Figure 4: Expressional changes in selected genes in MDA-MB-231 cells treated with different inhibitory concentrations of erufosine. Cells were treated with erufosine, and fold changes were calculated by using real-time PCR and Livak method. Expression levels of untreated controls were considered as base line.

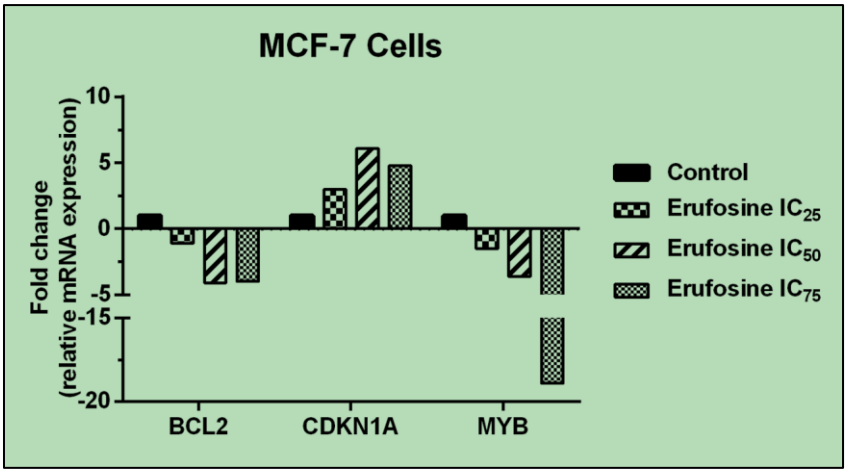

Figure 5: Expressional changes in selected genes in MCF-7 cells treated with different inhibitory concentrations of erufosine. Cells were treated with erufosine, and fold changes were calculated by using real-time PCR and Livak method. Expression levels of untreated controls were considered as base line.

DISCUSSION

Breast cancer is the most common cancer in females having different originated factors. Effective treatment strategies vary due to their different originating factors and molecular subtypes. Breast cancer can be treated by surgical removal, radiotherapeutic techniques and different available chemotherapeutic techniques. However, available treatments are not sufficient to cure the disease completely. Furthermore, several side-effects are also associated with current therapeutic agents. In this condition, it is important to identify novel anticancer compounds against breast cancer with a safer biological profile and greater effectiveness.

Erufosine is new synthetic alkyl-phospholipid (3rd generation) and has shown anticancer effects against variety of cancer cells. The compound interacts with cell membrane, especially the lipid-raft rich area and induces the subsequent anticancer effects. Previous generations of alkyl-phospholipids showed reasonable anticancer properties along with some side-effects. In comparison, erufosine is having properties of reduced gastrointestinal toxicity and hemolytic activity. This is a metabolically stable compound with potential of intravenous treatment and to cross blood-brain barrier. Erufosine forced anticancer impacts different cell lines by apoptosis, autophagy and G2 cell cycle. Erufosine also intercepted cholesterol homeostasis. Another significant characteristic of erufosine is the capability to induce apoptosis only in cancer cells. All these qualities strengthen the implementation of erufosine to accomplish pertinent clinical fixations, which was impractical with other ALPs. Erufosine is a highly toxic compound and can induce cytotoxic effects not only in primary and metastatic breast but also in other cancers including colorectal, liver and lung [5, 14-18].

As we know, anticancer agents impose their toxic effects via discrete signaling cascades. Considering

this, it is important to describe the cellular genetic expression being affected via erufosine for a clearer understanding about this anticancer compound. In the present study, effects of erufosine on expressional profiling of cell cycle, survival and apoptosis related genes in breast cancer cells were evaluated. As a first step, the selected cell lines (MDA-MB-231 and MCF-7) were cultured in 96-well plates and exposed to various concentrations of erufosine followed by assessment of viable cell fractions at three different time intervals (24, 48 and 72h). Cytotoxic effects of erufosine on these cell lines were determined by MTT dye reduction assay. The growth inhibition was maximum after 72h with 100μM concentrations and significant decline in cell survival rate was also observed with other erufosine concentrations for different time intervals. Overall, the effects were concentration dependent as cell death increased with increasing concentrations of erufosine. Interestingly, triple negative breast cancer cells (MDA-MB-231) were almost two time more responsive (IC50: 20μM, 72h) as compared to receptor positive cells (MCF7, IC50: 40 μM, 72h).

CDKN1A, which is a well-known cell cycle inhibitor, while BCL2 is an anti-apoptotic gene, while third gene (MYB) play multiple roles and affects cell survival and differentiation (19-21). In cancer cells, these genes are often de-regulated and facilitate the progression of cancer cells. To evaluate effects of erufosine on these genes, we designed the primers of genes (BCL2, CDKN1A, MYB) and observed that erufosine induces substantial expression modulations in these genes. Precisely, the compound induced the cell cycle inhibitor (CDKN1A) in the two cell lines. This indicates that erufosine can halt the cell cycle machinery and is a cytostatic agent. On other side, erufosine inhibited the MYB gene, which means tumor cell survival and differentiation is compromised after the treatment with erufosine. These effects were clear and comparable in the two breast cancer cell lines representing the different molecular subtypes. As far as BCL2 (a pro-apoptotic) gene is concerned, it was inhibited in MCF-7 cells, while induced in MDA-MB-231 cells at lower concentrations of erufosine. This indicates that triple negative cells (MDA-MB-231) showed resistance towards lower concentration of erufosine and tried to avoid apoptosis.

Overall, erufosine induces substantial toxic effects in breast cancer cells irrespective of their molecular subtype. The compound has the potential to alter the expression of multiple genes in breast cancer cells. Based on current study, erufosine seems to be a potential antineoplastic agent and it is recommended to expand the study by further *in vitro* and *in vivo* studies.

REFERENCES

1. Vink SR, Schellens JH, van Blitterswijk WJ, Verheij M. Tumor and normal tissue pharmacokinetics of perifosine, an oral anti-cancer alkylphospholipid. Investigational new drugs. 2005 Aug;23:279-86.
2. Berger MR, Sobottka S, Konstantinov SM, Eibl H. Erucylphosphocholine is the prototype of iv injectable alkylphosphocholines. Drugs of today. 1998;34:73-81.
3. van Blitterswijk WJ, Verheij M. Anticancer mechanisms and clinical application of alkylphospholipids. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2013 Mar 1;1831(3):663-74.
4. Jendrossek V, Hammersen K, Erdlenbruch B, Kugler W, Krügener R, Eibl H, Lakomek M. Structure-activity relationships of alkylphosphocholine derivatives: antineoplastic action on brain tumor cell lines in vitro. Cancer chemotherapy and pharmacology. 2002 Jul;50:71-9.
5. Berger MR, Tsoneva I, Konstantinov SM, Eibl H. Induction of apoptosis by erucylphospho-N, N, N-trimethylammonium is associated with changes in signal molecule expressionand location. Annals of the New York Academy of Sciences. 2003 Dec;1010(1):307-10.
6. Kaleağasioğlu F, Berger MR. Differential effects of erufosine on proliferation, wound healing and apoptosis in colorectal cancer cell lines. Oncology Reports. 2014 Mar;31(3):1407-16.
7. van Blitterswijk WJ, Verheij M. Anticancer mechanisms and clinical application of alkylphospholipids. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2013 Mar 1;1831(3):663-74.
8. Bagley RG, Kurtzberg L, Rouleau C, Yao M, Teicher BA. Erufosine, an alkylphosphocholine, with differential toxicity to human cancer cells and bone marrow cells. Cancer chemotherapy and pharmacology. 2011 Dec;68:1537-46.
9. Dineva IK, Zaharieva MM, Konstantinov SM, Eibl H, Berger MR. Erufosine suppresses breast cancer in vitro and in vivo for its activity on PI3K, c-Raf and Akt proteins. Journal of cancer research and clinical oncology. 2012 Nov;138:1909-17.
10. Königs SK, Pallasch CP, Lindner LH, Schwamb J, Schulz A, Brinker R, Claasen J, Veldurthy A, Eibl H, Hallek M, Wendtner CM. Erufosine, a novel alkylphosphocholine, induces apoptosis in CLL through a caspase-dependent pathway. Leukemia research. 2010 Aug 1;34(8):1064-9.
11. Zaharieva MM, Kirilov M, Chai M, Berger SM, Konstantinov S, Berger MR. Reduced expression of the retinoblastoma protein shows that the related signaling pathway is essential for

mediating the antineoplastic activity of erufosine. PLoS One. 2014 Jul 2;9(7):e100950.
12. Yosifov DY, Dineva IK, Zaharieva MM, Konstantinov SM, Berger MR. The expression level of the tumor suppressor retinoblastoma protein (Rb) influences the antileukemic efficacy of erucylphospho-N, N, N-trimethylpropylammonium (ErPC3). Cancer biology & therapy. 2007 Jun 1;6(6):930-5.
13. Konstantinov SM, Georgieva MC, Topashka-Ancheva M, Eibl H, Berger MR. Combination with an antisense oligonucleotide synergistically improves the antileukemic efficacy of erucylphospho-N, N, N-trimethylpropylammonium in chronic myeloid leukemia cell lines. Molecular Cancer Therapeutics. 2002 Aug 1;1(10):877-84.
14. Rudner J, Ruiner CE, Handrick R, Eibl HJ, Belka C, Jendrossek V. The Akt-inhibitor Erufosine induces apoptotic cell death in prostate cancer cells and increases the short term effects of ionizing radiation. Radiation Oncology. 2010 Dec;5:1-2.
15. Ansari SS, Akgün N, Berger MR. Erufosine increases RhoB expression in oral squamous carcinoma cells independent of its tumor suppressive mode of action-a short report. Cellular oncology. 2017 Feb;40:89-96.
16. Nasim M, Batool A, Kanwal K. Cytotoxic effects and expressional modifications induced by alkyl-phospholipid (erufosine) in lung cancer cells. Cancer Research and Medicine. 2024 Dec 26;1(1).
17. Shafiq N, Iqbal UU, Javed A, Pervaiz A. Effects of a synthetic alkyl-phospholipid on transcriptomic profile of cell cycle genes in liver cancer cells. Biomedical Current Insights. 2025 Jun 11;2(1).
18. Kanwal K, Batool A, Nasim M. Cytotoxic effects of alkyl-phospholipids (Erufosine and Perifosine) in combination with cell cycle check point inhibitor (ATM) in breast cancer cells. Biomedical Current Insights. 2024 Dec 28;1(1).
19. Cazzalini O, Scovassi AI, Savio M, Stivala LA, Prosperi E. Multiple roles of the cell cycle inhibitor p21CDKN1A in the DNA damage response. Mutation Research/Reviews in Mutation Research. 2010 Apr 1;704(1-3):12-20.
20. Siddiqui WA, Ahad A, Ahsan H. The mystery of BCL2 family: Bcl-2 proteins and apoptosis: an update. Archives of toxicology. 2015 Mar;89(3):289-317.
21. Ramsay RG, Gonda TJ. MYB function in normal and cancer cells. Nature Reviews Cancer. 2008 Jul;8(7):523-34.

Authors Contribution: ARA was responsible for experimental design, execution and data analysis. TS was responsible for literature review and manuscript preparation.
Funding: No specific grant was received for the work.
Competing Interests: No competing interests.
Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.