EVALUATION OF PRO-APOTOTIC EFFECTS OF β-MONOLINOLEIN ON METASTATIC BREAST CANCER CELL LINE MDA-MB-231

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ABSTRACT

Objectives: The objective of this study was to evaluate the β-monolinolein as a potential therapy for breast cancer treatment.

Methods: The cytotoxic activity of β-monolinolein was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and trypan blue exclusion assay. The cellular cytotoxicity and levels of cytosolic enzyme lactate dehydrogenase (LDH), were measured by assessing umoles of nicotinamide adenine dinucleotide/well/min. To confirm whether β-monolinolein induces apoptosis in 3,4-methylenedioxyamphetamine (MDA)-MB-231 cells, western blot and semi quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis were performed.

Results: For the 1st time, it was demonstrated that β-monolinolein strongly inhibits the growth of MDA-MB-231 cells, with an half maximal inhibitory concentration value of 12.5 µg/ml. <90% of cell death was achieved at higher concentrations after 48 h of treatment. Trypan blue assay showed that the cell viability was significantly decreased in a dose-dependent manner in MDA-MB-231 cells after 48 h of treatment. On the other hand, LDH activities in the cultured media were significantly elevated in a dose-dependent manner as compared to the control. Further, the western blot analysis showed that β-monolinolein leads to change in expression levels of important cell cycle regulators such as p21, Bax, Bcl-XL, and Bcl-2 in MDA-MB-231 cells. The semi quantitative RT-PCR results indicated a significant upregulation of proapoptotic genes such as p53, p21, and Bax and downregulation of antiapoptotic gene Bcl-2.

Conclusion: These results indicate that β-monolinolein leads to change in expression of various cell cycle/apoptotic regulators and hence induces death in MDA-MB-231 cells.

Keywords: β-monolinolein, Cytotoxicity, Breast cancer treatment.

INTRODUCTION

Cancer is defined as a complex series of life-threatening disease condition and a leading cause of death caused by persistent tissue injury and host-environment interactions [1]. Worldwide, cancer is known to be the second leading cause of death and is responsible for an estimated 9.6 million deaths in 2018. Globally, about one in six deaths are due to cancer [2]. The repeated exposure of carcinogens such as tobacco, ultraviolet light, and infections leads to various genetic (mutations), epigenetic (loss of heterozygosity), and global transcriptome changes (through inflammation pathways) and is associated with increased cancer risk [3]. Among cancers, breast cancer is projected to be among the most common cancer and is the most frequent malignant neoplasm in women [4]. Due to increased occurrence of cancer and worldwide prevalence during the past decade, it has posed a great challenge to the health-care professionals. The latest WHO statistics suggests about 45% increase in the global cancer deaths by 2030, of which 70% would be contributed from developing countries like India [5]. Despite a better understanding of disease and the advent of modern technology and rationally targeted drugs, the incidence and cure rate of cancer have not improved. Cancer cure and prevention, therefore, remain a high priority for scientific community across the world [6].

Although a range of conventional therapies based on chemotherapy, surgery, and radiotherapy is available, these approaches are in many cases of limited efficacy [7]. Moreover, the current anticancer regimens are frequently associated with significant levels of toxicity and the emergence of drug resistance. One major challenge to relieve cancer burden is to develop highly effective drugs with specificity on cancers but little or no side effects on normal mammalian cells [8]. Many research projects have been focused on developing novel chemotherapies either by exploring the anticancer ability of novel compounds or by assessing drugs conventionally used in other clinical diseases. Hence, the search for cancer treatment continues to be a worldwide effort. As part of this effort, various natural products, especially from medicinal plants, have been tested against various cancer cell lines [9,10]. Still, there has been a long-standing interest in the identification of medicinal plants and derived natural products for developing novel cancer therapeutics [11,12]. Natural products have been playing a major role in the search for novel drugs for numerous illnesses including cancer [13,14]. Throughout human history and especially in the past century, natural products have virtually remained undisputed leaders among the various therapeutic tools humans have employed against many diseases including cancer [15]. Natural products have been found to be a relevant source of novel and potent bioactive compounds with minimal side effects in vivo. Many phytochemicals used for the treatment of malignant tumors are cytotoxic drugs that can induce tumor cell death by apoptosis [16]. Chemotherapy with cytotoxic drugs is the main treatment modality for certain types of cancer [17]. In vitro cell-based assays have been developed to rapidly determine the cytotoxic activity of several compounds. Cell-based assays are also useful in identifying variations in susceptibility of different target cells to several chemotherapeutic agents [18,19]. The specific intracellular damage induced by these drugs is generally well characterized. For example, cisplatin, one of the most widely used anticancer drugs, works with the target genomic DNA to form DNA adducts [20]. This DNA damage can induce cell cycle arrest and apoptosis [21,22]. Inhibition of proliferation and/or induction of apoptosis in cancer cells are the most important characteristic of many anticancer agents [23]. However, the mechanisms by which the specific damage induced by the chemotherapeutic agents...
that are converted into a death signal remain poorly understood. Breast cancer has been found attenuated by an appreciable amount of natural substances including phytochemicals and dietary substances which affect cell proliferation, cell differentiation, angiogenesis, apoptosis, and a few other cellular transduction pathways [24]. The increasing global incidence of breast cancer emphasizes the need to understand the mechanism involved in breast tumorigenesis and to identify novel, safe, and efficient anticancer drugs for the treatment of breast cancer. Nevertheless, the continued search for safer and more effective natural agents to improve the efficiency of breast cancer treatment is furthermore a need [25].

Despite the reports on the biological activities of these essential oils, data on their cytotoxicity are still scarce in the literature [26,27]. Discovery and development of new chemopreventive drugs against breast cancer with an interesting safety and efficacy to improve breast cancer management and reduce the high cost and pain of patients are an urgent necessity [28]. Hence, the objective of the present study is to evaluate the cytotoxic and proapoptotic activity and potency of β-monolinolein on metastatic breast cancer cell line 3,4-methylenedioxyamphetamine (MDA)-MB-231.

**METHODS**

**Drug preparation**

β-monolinolein (9,12-octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester) was isolated, identified, and characterized from the leaves of Abrus precatorius L. using various techniques such as Soxhlet extraction, thin-layer chromatography, column chromatography, high-performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR), and by gas chromatography–mass spectrum analysis, as discussed in our published research work [29].

**Cell culture and maintenance**

MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

**(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cytotoxicity**

Analysis of cell viability was done by standard colorimetric MTT assay. After 24 h, cells were treated with different concentrations of β-monolinolein for 48 h. 20 μl of MTT (5 mg/ml) reagent was added to each well and then incubated for 3 h at 37°C in CO₂ incubator [18]. After 3 h, the medium was removed and 200 μl of dimethyl sulfoxide (DMSO) was added and mixed. Absorbance was recorded at 595 nm with multiwell-plate reader. The percentage of cell viability of β-monolinolein was calculated according to the following equation: Percentage of cell viability = (OD of treated cells/OD of control cells) ×100. The concentration of drug that inhibits 50% of the cells ([IC₅₀] values) for these samples was obtained from dose-response curves [30].

**Trypan blue dye exclusion assay**

For the determination of cell viability, MDA-MB-231 breast cancer cells were plated at a density of 1×10⁵ cells/well in 24-well tissue culture plates and cultured for 48 h at 37°C. The medium was replaced with serum-free medium and cells were treated with various concentrations of β-monolinolein (10, 20, 30, 40, and 50 μg/ml) for a further 48 h. The cultures were harvested and washed twice with phosphate-buffered saline (PBS), cell pellet was then suspended with 0.5 ml PBS. Then, 20 μl of cell was mixed with equal volume of 0.4% trypan blue (Sigma-Aldrich, USA). The number of live/dead cells was directly counted on hemocytometer under microscope. The percentage viability for the cultures was calculated as (live cells/total cells) ×100.

**Lactate dehydrogenase (LDH) cytotoxicity assay**

MDA-MB-231 cells (1×10⁵ cells/well) were plated in 100 μl of medium per well in 96-well plates and were allowed to attach to the plate for 24 h. After cell attachment (24 h), cells were treated with increasing concentrations of β-monolinolein (10, 20, 30, 40, and 50 μg/ml) for a further 48 h. The extracellular LDH activity was measured in the medium after 24 h. Following treatment, culture supernatants were then collected from each well and transferred to individual tubes containing Tris-ethylenediaminetetraacetic acid-nicotinamide adenine dinucleotide (NADH) buffer followed by 10 min incubation at 37°C and the addition of pyruvate solution. Absorbance was read at 339 nm using a ultraviolet (UV)-visible spectrophotometer (UV-260, Shimadzu Corp, Japan). LDH activity was expressed as μmoles of NADH used per minute per well. All experiments were repeated 3 times in triplicates.

**Western blot analysis**

Change in the protein levels of various cell cycles/apoptotic regulators such as p21, BAX, Bcl-XL, and BCI-2 was analyzed by western blot analysis. Specific antibodies to p21, BAX, Bcl-XL, BCI-2, and β-actin (loading control) were used. The membranes were washed with Tris-Buffered Saline Tween-20 and incubated with specific secondary antibodies. Femtolucent substrate (G-biosciences, USA) was used to detect the antibodies by exposing the blot to an X-ray film. Developer and fixer were purchased from Eastman Kodak (USA).

**Gene expression analysis by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)**

Expression level of various apoptosis-related genes was analyzed by semiquantitative RT-PCR. For expression analysis, gene-specific primers were used as listed in Table 1.

**Statistical analysis**

The data represent the mean ± standard deviation of three independent experiments each in a triplicate. The significance between control and treated groups was analyzed by Student’s t-test and p<0.05 was taken as statistically significant by GraphPad Prism 5.0 Software (GraphPad Software Inc, CA, USA).

**RESULTS**

**MTT assay for cytotoxicity**

Treatment of MDA-MB-231 cells for 48 h with β-monolinolein showed a very significant and higher cytotoxic activity with an IC₅₀ value of 12.5 μg/ml (Fig. 1). These results indicated that β-monolinolein has a potent cytotoxic activity on MDA-MB-231 breast cancer cells.

**Morphological studies**

Microscopic examination of MDA-MB-231 cells treated with β-monolinolein showed significant morphological changes such as shrinkage and detachment from the surface (Fig. 2). Due to the loss of cells, differences in the cell number can also be visualized in culture dish when it was treated with β-monolinolein for 48 h in comparison to the control dish. These results show that β-monolinolein has a potent cytotoxic activity on breast cancer cells.

**Table 1: Amplicon size and sequence of the primers used in semiquantitative RT-PCR**

| Sl. No | Gene | Amplicon size | Primer sequence |
|-------|------|---------------|-----------------|
| 1.    | p21  | 147 bp        | Forward: GCAATTAGGGCATCACAGT<br>Reverse: TGGCTTCCAGTCTGGTTCTCTG |
| 2.    | BID  | 133 bp        | Forward: TGTTGATGCACTCATCCTGGT<br>Reverse: CCAATAGGAAGAAAGCCTGA |
| 3.    | BAX  | 132 bp        | Forward: CCTAGGGGATCCCTCTG<br>Reverse: GCTGGATCCAAACAGCACA |
| 4.    | Bcl-2| 141 bp        | Forward: CGAGGAGGGAGTGAGGAC<br>Reverse: TGATGTGAGTCTGGTGAGG |
| 5.    | Rpl35a| 141 bp       | Forward: CTTGGTTTTCTTTGTTG<br>Reverse: AAGGGAGGACACAGGCTTC |

RT-PCR: Reverse transcription-polymerase chain reaction
Trypan blue dye exclusion assay
Direct counting for non-viable and viable cells using trypan blue exclusion test showed that β-monolinolein treated cells with the different concentrations ranging from 10 to 50 µg/ml for 48 h reduced the cell viability in dose-dependent manner (Fig. 3). At 10 µg/ml viability was decreased to 62.41%, whereas at 50 µg/ml viability reached to minimum value of 11.84%. This assay showed that β-monolinolein killed human breast cancer cells at a higher rate than control (without DMSO) and DMSO. The data suggest that β-monolinolein treatment results in dose-dependent growth inhibition and induces cell death in MDA-MB-231 cells.

LDH cytotoxicity assay
LDH level was increased from 1.0 µmoles (control) to 8.83 µmoles (treated at 50 µg/ml of extract) of NADH. Cells treated with β-monolinolein for 48 h resulted in a significant increase (7.83-fold) in LDH release relative to the untreated cells in dose-dependent manner (Fig. 4). Cytotoxic effects of β-monolinolein due to membrane damage leads to cytosolic LDH release could be one of the reasons, leading to cell death.

Western blot analysis
Western blot analysis shows that β-monolinolein treatment leads to upregulation of proapoptotic proteins such as p21 and Bax. Downregulation of antiapoptotic proteins Bcl-XI and Bcl-2 in MDA-MB-231 cells was also determined after treatment with β-monolinolein (Fig. 5a). As shown in Fig. 5b, the treatment leads to a 9-fold upregulation of p21, 3-fold upregulation of Bax, 5-fold downregulation of Bc-XL, and 6–7-fold downregulation of Bcl-2 at 20 µg/ml concentration of β-monolinolein. Together these results show that β-monolinolein has a potent effect on expression of various regulators of the cell cycle and apoptosis.

Gene expression analysis by semiquantitative RT-PCR
Semiquantitative RT-PCR analysis indicates a significant upregulation of proapoptotic genes such as p21, BID, and Bax on treatment with...
β-monolinolein. In addition, downregulation of antiapoptotic gene Bcl-2 was also observed (Fig. 6a). The graphical representation of the quantitative analysis of RT-PCR results was shown in Fig. 6b. Treatment of MDA-MB-231 cells with β-monolinolein leads to approximately 8–9-fold upregulation of p21, 5-fold upregulation of BID, and 3–4-fold upregulation of BAX. In the same way, around 4–5-fold downregulation of antiapoptotic gene Bcl-2 was quantified after treatment with β-monolinolein at concentration of 20 μg/ml. These results indicate that β-monolinolein leads to change in expression of various cell cycle/apoptotic regulators and hence induces death in MDA-MB-231 cells.

**DISCUSSION**

Medicinal herbs have been widely accepted with escalating awareness all over the world. Nowadays, about 65% of plant-based medicines are used for cancer therapy [31]. Clinically used anticancer drugs generally induce complex responses in cancer cells [32]. Studying the mechanisms underlying these responses is important to understand how drugs should be optimally used and why tumor cells become resistant to them. Various forms of cancers require multiple approaches for their treatment, which opens a wide field of research in the discovery of new anticancer natural products [33]. Hence, it is important to study novel mechanisms of selected cytotoxic compounds action that could potentially represent new weapons in the fight against cancer [34]. Therefore, the continued research in this field appears to hold great promise for newer therapeutic molecules to treat cancer. Medicinal plants are the richest bioresource of drugs in traditional medicine, pharmaceutical intermediates, and chemical entities for synthetic drugs [35]. Medicinal plant extracts usually contain many secondary metabolites with varying bioactivities [36]. Several bioactive compounds have been discovered in plants and used directly as patented drugs such as taxol, artemisinin, and maprouneacin [37]. Cytotoxicity screening models provide important preliminary data to select plant extracts with potential anticancer properties [38]. In our published research work, it was found that the leaf extract of *Abrus precatorius* is known to consist of cytotoxic compounds that are very effective against metastatic breast cancer MDA-MB-231 cells [39]. Further, two anticancer compounds β-monolinolein and stigmasterol hemihydrate were isolated from the leaves of *A. precatorius* by various bioassay-guided isolation and characterization techniques [29]. Both these compounds were experimentally proved to be having chemopreventive

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**Fig. 5:** Western blot analysis showing change in expression of various apoptotic and cell cycle regulators in 3,4-methylenedioxyamphetamine-MB-231 cells.  
(a) Lane 1=Untreated; Lane 2–4=Cells treated with indicated concentrations of β-monolinolein, after 48 h of treatment.  
(b) Graphs show the densitometry quantitation of the proteins indicated in the western blots

**Fig. 6:** Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of various cell cycle regulators,  
(a) Lane 1 and 2 =Untreated; Lane 3–8=Cells treated with indicated concentrations of β-monolinolein, (b) The graphs represent the change in expression of the indicated genes as quantitated by densitometry analysis of the semiquantitative RT-PCR products
and anti-breast cancer activity both in vitro and in vivo experimental models. Now, in the present study, β-monolinolein that was having maximum cytotoxic activity was tested separately for its cytotoxic and pro-apoptotic activity and for molecular mechanism behind its activity on metastatic breast cancer cell line MDA-MB-231.

The β-monolinolein is long-chain unsaturated fatty acids. It was previously reported that fatty acid esters are known to exhibit cytotoxicity against HeLa, HepG2, and MCF-7 cells [40,41]. In the present investigation, for the 1st time, it was demonstrated that β-monolinolein was found to be significantly active and inhibits growth of MDA-MB-231 cells, with an IC₅₀ value of 12.5 μg/ml. More than 90% of cell death was achieved at higher concentrations after 48 h of treatment. Microscopic examination of MDA-MB-231 cells treated with β-monolinolein showed significant morphological changes such as shrinkage and detachment from the surface. Due to the loss of cells, differences in the cell number can also be visualized in culture dish when it was treated with β-monolinolein for 48 h in comparison to the control dish. These results show that β-monolinolein has a potent cytotoxic activity on breast cancer cells.

Further, trypan blue exclusion assay showed the reduction of cell viability in concentration-dependent manner in treated MDA-MB-231 cells as compared to the control cells. Another parameter for cell death is the integrity of the cell membrane which can be measured by the cytoplasmic enzyme released by damaged cells. LDH is a stable cytoplasmic enzyme abundant in the cytosol of all mammalian cells. It is rapidly released into the cell culture supernatant on damage of the plasma membrane [42]. The cytotoxicity induced by β-monolinolein was assessed by LDH leakage into the culture medium. Cell membrane rupture was defined as the ratio of LDH activity in the supernatant of treated cells to LDH activity released in the control cells. The released LDH levels were significantly elevated after 48 h of β-monolinolein treatment in the medium in comparison to the non-treated cells.

The western blot results showed that β-monolinolein leads to upregulation of p21 and Bax proteins in a concentration-dependent manner and downregulation of Bcl-XL and Bcl-2 in treated cells. Collectively the results proved that β-monolinolein has a very prominent effect on the expression levels of critical regulators of the cell cycle and apoptosis. Several studies have shown that phytochemicals such as curcumin, resveratrol, flavopiridol, indole-3-carbinol, ecdysone, and green tea polyphenols are also known to induce apoptosis by downregulating expression of Bcl-2 and Bcl-XL in several cancer cell lines [43]. Cell cycle progression is controlled by the expression of various genes [44]. The balance between the expression of pro-apoptotic and anti-apoptotic genes is important to determine whether cell would survive or die. Various unfavorable conditions alter the steady-state levels of these genes resulting in programmed cell death [45]. The increase in the expression of apoptotic genes leads to the release of cytochrome C from the mitochondria, which triggers activation of caspases that, in turn, lead to apoptosis of the cells [46].

The results showed that the treatment of MDA-MB-231 cells with the β-monolinolein regulates the expression of key cell cycle mediators in MDA-MB-231 cells. RT-PCR results indicated a significant upregulation of pro-apoptotic genes such as p53, p21, and Bax and downregulation of anti-apoptotic gene Bcl-2. These genes have also been demonstrated to play an important role in initiation and execution of apoptosis in tumors exposed to radiation or chemotherapy. The Bcl-2 family proteins are well known to be decisive regulators in promoting cell death [47].

**CONCLUSION**

Our findings suggest that β-monolinolein might prove to be a potential source of anticancer lead molecule. However, further, molecular work is still needed to validate β-monolinolein for possible anticancer drug development and the clinical purpose.

**CONFLICTS OF INTEREST**

We declare that we have no conflicts of interest.

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