Molecular mechanisms of anticancer activity of deoxyelephantopin in cancer cells

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ABSTRACT

Background: Deoxyelephantopin (DOE) is a natural bioactive sesquiterpene lactone from Elephantopus scaber, a traditionally relevant herb in Chinese and Indian medicine. It has shown promising anticancer effects against a broad spectrum of cancers.

Methods: We examined the effect of DOE on growth, autophagy, apoptosis, cell cycle progression, metastasis, and various molecular signaling pathways in cancer cells, and endeavored to decipher the molecular mechanisms underlying its effect. The cytotoxicity of DOE was examined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and colony formation assays. The antimitastatic potential of DOE was identified by wound closure, as well as invasion and migration assays. The expression of mRNAs and proteins related to cytotoxicity in cancer cells induced by DOE was investigated using reverse transcription-polymerase chain reaction, flow cytometry, and Western blot analysis.

Results: DOE showed significant cytotoxicity and induced apoptosis in cancer cells. DOE promoted the autophagy of HCT 116 and K562 cells. DOE arrested cell cycle progression in the G2/M phase. DOE treatment caused activation of caspase-8, -9, -3 and -7, reactive oxygen species production, and cleavage of cleavage of poly-ADP-ribose polymerase (PARP), the markers of apoptosis. Moreover, apoptosis induction was associated with mitochondrial permeability and endoplasmic reticulum stress. Treatment of cancer cells with DOE inhibited mitogen-activated protein kinases, nuclear factor-kappa B, phosphatidylinositol 3-kinase (PI3K/Akt), and β-catenin signaling. Furthermore, treatment of DOE increased the expression of p53, phospho-Jun amino-terminal kinases (p-JNK), and p-p38 and decreased the expression of phospho-signal transducer and activator of transcription 3 (p-STAT3) and phospho-mammalian target of rapamycin (p-mTOR) in cancer cells. DOE downregulated matrix metalloproteinase (MMP-2) and MMP-9, urokinase-type plasminogen activator (uPA), and urokinase-type plasminogen activator receptor (uPAR) mRNA levels in cancer cells.
Conclusion: These findings concluded that DOE may be useful as a chemotherapeutic agent against cancer.

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1. Introduction

Cancer is the second most common disease after cardiovascular disorders worldwide. Death by cancer is mainly attributable to metastasis that cannot be cured with current treatments. Surgery, radiotherapy, and chemotherapy are the major treatment modalities for cancers. Toxicity to normal cells and multidrug resistance remain a challenging issue in cancer treatment. Therefore, it is necessary to develop new anticancer drugs with better efficacy and low incidence of side effects.

The modes of cell death induced by chemotherapeutic drugs include apoptosis, necrosis, and autophagy. Autophagy, a process of homeostasis, is highly correlated with drug-induced cell death. Apoptotic processes could be triggered via either extrinsic pathways or intrinsic pathways. The mitochondria-mediated intrinsic pathway of apoptosis is mainly regulated by the interaction between the Bcl2 family of proteins that comprise proapoptotic, antiapoptotic, and proapoptotic BH3-only proteins. The inhibitor of apoptosis (IAP) family constitutes a family of proteins that can inhibit apoptosis by binding and inhibiting active caspasas. Recent studies have suggested the role of the endoplasmic reticulum (ER) in chemotherapy-induced cytotoxicity. p53, a well-known cancer suppressor, acts as a transcription factor that regulates cell cycle arrest and induces apoptosis via upregulation of Bcl2 family proteins. Reactive oxygen species (ROS) in cancer cells lead to severe and irreversible oxidative damage, dysfunction of mitochondria, and release of cytochrome c, which in turn activates caspase-3 initiating mitochondria/cytochrome c-mediated apoptosis.

Targeted therapies against signaling pathways involved in cell proliferation, metastasis, apoptosis, and cell cycle regulation may provide a new insight into cancer therapeutics. Most of the chemotherapeutic drugs exert their anticancer activity by altering the cell cycle progression. Constitutive activation of mitogen-activated protein kinases (MAPKs) is often associated with carcinogenesis. c-Jun N-terminal kinase (JNK) and p38 are stress-related kinases, and their activation is associated with apoptotic induction, whereas extracellular signal-regulated kinase 1/2 (ERK1/2) activation is antiapoptotic. Phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling is considered to be a promising drug target in the development of cancer chemotherapeutic agents. As many of downstream targets of Wnt/β-catenin signaling pathway play an important role in tumorigenesis, the regulation of these components is believed to be a rational target for cancer therapy. Signal transducer and activator of transcription 3 (STAT3), a well-recognized protein in the STAT family, has gained attention in cancer therapy because of its persistent activation in a variety of human cancers.

Phytochemicals derived from herbal medicines are found to be potent anticancer and antimetastatic agents. Deoxyelephantopin (DOE), a sesquiterpene lactone from Elephantopus scaber, exerts many beneficial effects including hepatoprotective, antiprotozoal, and wound healing. DOE has been reported to have anticancer effect. The present study was designed to elucidate underlying mechanisms of action of DOE in cancer cells.

2. Methods

2.1. Chemicals

Dulbecco’s modified Eagle’s medium (DMEM), propidium iodide (PI), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), Acridine Orange (AO), ethidium bromide (EB), N-acetyl-L-cystein (NAC), and 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin and fetal bovine serum (FBS) were supplied by Pan Biotech, St. Louis, MO, USA. Crystal Violet and dimethyl sulfoxide were procured from Merck (Mumbai, India).

2.2. DOE

DOE (Fig. 1A, Table S2), isolated from E. scaber as previously described, was dissolved in dimethyl sulfoxide (10 mg/mL) and stored at −20 °C as stock solution and was diluted to the required concentration immediately prior to use with DMEM.

2.3. Cell culture

HCT 116 (colorectal), K562 (chronic myeloid leukemia), KB (oral), and T47D (breast) cancer cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM supplemented with 10% FBS, streptomycin (100 μg/mL), and penicillin (100 U/mL), and maintained in a humidified atmosphere at 37 °C and 5% CO2.

2.4. Cytotoxicity assay

Briefly, 5 × 103 cells/100 μL/well were seeded in 96-well plates and incubated overnight at 37 °C in a humidified incubator and 5% CO2. The cells were treated with various concentrations of DOE and further incubated for 24 hours, 48 hours, and 72 hours. Next, 100 μL of MTT (5 mg/mL) was added to each well and incubated at 37 °C for 2 hours in the dark. Lysis buffer (100 μL) was added and further incubated at 37 °C for 4 hours. The absorbance at 570 nm was recorded by a microplate reader (BioTek Instruments, Winooski, VT, USA).
Fig. 1 – DOE inhibited the cell growth and colony formation of cancer cells. (A) Structure of DOE. (B) Cancer cells were treated with DOE and MTT assay was performed. (C) IC\textsubscript{50} values of DOE after 48 hours of exposure. (D) In colony forming assay, cancer cells were seeded after treatment with DOE. (E) In soft agar colony forming assay, K562 cells were seeded after exposure to DOE into 0.3% agar in six-well plates. The plates were photographed and colonies were counted after 14 days. Data are presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control.

DOE, deoxyelephantopin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SD, standard deviation.
2.5. Colony formation assay

Cancer cells pretreated with DOE for 2 hours were washed, seeded in six-well plates (400 cells/well), and incubated for 2 weeks. The colonies were fixed, then stained with 0.1% crystal violet for 10 minutes. The number of colonies (more than 50 cells/colony) was counted under an inverted microscope.

2.6. Soft agar colony formation assay

In six-well plates, K562 cells (400 cells/well), with or without treatment with DOE, were seeded in the upper layer containing 0.7% agar, DMEM, and 10% FBS. The bottom agar base (lower layer) contained 1% agar supplemented with 20% FBS. After 14 days of incubation, colonies were visualized by 0.03% crystal violet staining, and the number of colonies was counted.

2.7. Fluorescent microscopy

The morphological changes of cells were observed under a light microscope after treatment with DOE for 48 hours. Cells (2 × 10⁴/well) were incubated with DOE for 48 hours and stained with AO–EB dye and Hoechst 33342 stain. Cells were viewed under a fluorescence microscope (Olympus 1X 51; Olympus Corp., Tokyo, Japan).

2.8. Fluorescein isothiocyanate–annexin V and PI assay

After treatment with DOE, 2 × 10⁵ cells were collected, washed twice with cold phosphate-buffered saline (PBS), and then suspended in 1× binding buffer. Cells were stained with annexin V/PI, incubated for 15 minutes, and then analyzed by flow cytometry.

2.9. DNA fragmentation analysis

After incubation with DOE, cells were washed with PBS and suspended in 500 µL DNA extraction buffer. Proteinase K (1 mg/mL) was added and incubated overnight in a water bath. DNA was precipitated by adding isopropanol, and the tubes were kept at −20°C for 30 minutes followed by centrifugation (11,260g for 20 minutes at 4°C). DNA was subjected to electrophoresis on 1.5% agarose gel.

2.10. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed according to the manufacturer’s protocols (Promega, Madison, WI, USA). Cells (2 × 10⁵/well) were exposed to DOE for 48 hours, fixed with 4% formaldehyde in PBS, and treated with 0.2% Triton X-100 in PBS for 5 minutes. Equilibration buffer (100 µL) was added followed by incubation with TdT reaction buffer for 1 hour in the dark. TUNEL-positive cells were evaluated by fluorescence microscopy.

2.11. Measurement of intracellular ROS level

Cancer cells (20 × 10⁵) were seeded in black 96-well plate and incubated overnight. The next day, the medium was replaced with indicated concentration of DOE. At the end of incubation, the supernatant was discarded and the cells were rinsed with PBS for 10 minutes. The cells were incubated with H2DCF-DA (20 µM) in PBS for 30 minutes at 37°C. Cells were washed twice in PBS, and fluorescence acquisition was done in a plate reader. In addition, ROS production was monitored using fluorescence microscopy. To determine whether intracellular ROS levels play any role in the cytotoxicity of DOE, tumor cells were pretreated with NAC (5 mM) for 2 hours prior to treatment with DOE in 96-well plate at 37°C and 5% CO₂. After treatment with DOE, ROS was measured. The MTT assay was used to measure the cytotoxicity of DOE in the presence of NAC.

2.12. Cell cycle analysis

Cells (1 × 10⁶), after 48 hours of treatment with DOE, were fixed in ice-cold 70% ethanol overnight. The cells were washed in ice-cold PBS and incubated with PI and RNase A for 30 minutes. Samples were analyzed using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.13. Caspase-3 detection by flow cytometry

The activity of caspase-3 was determined using fluorescein isothiocyanate-conjugated anticaspase-3 antibody kit (BD Biosciences) according to the manufacturer’s protocol. The viability of cells pretreated with a general caspase inhibitor z-VAD-fmk after DOE treatment for 48 hours was checked using the MTT assay.

2.14. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from DOE treated cancer cells using TRI Reagent (Sigma, St. Louis, MO, USA), and cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instruction. The primers used in reverse transcription-polymerase chain reaction (RT-PCR) are presented in Table S1. PCR products were separated on a 2% agarose gel and visualized under UV light by EB staining.

2.15. Western blotting

DOE-treated cells (1 × 10⁶) were lysed in a protein extraction buffer and centrifuged at 11,260g for 20 minutes at 4°C. The supernatant was collected and concentration of protein was quantified by Bradford protein assay (Merck, India). Proteins (50 µg/well) were electrophoresed on 8% sodium dodecyl sulfate–polyacrylamide gel, and resolved proteins were transferred onto polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% milk–TBST (Tris-buffered saline with 0.05% Tween 20) for 1 hour. The membranes were probed with various primary antibodies (1:1000) against procaspases (-3, -8, -9, -7), cytochrome c, poly-ADP-ribose polymerase (PARP), p44/p42,
2.16. Statistical analysis

Data are shown as mean ± standard deviation. Significant differences were determined using unpaired Student t test (Graph Pad Prism software Inc., San Diego, CA, USA). A p value less than 0.05 was considered statistically significant.

3. Results

3.1. DOE inhibited growth and colony forming property of cancer cells

In this study, the effect of DOE was evaluated for in vitro cytotoxicity against cancer cells. As shown in Fig. 1B, the cell death rate increased with the increase in concentration and incubation time of DOE across all four-cell lines. The obtained results indicated that DOE was shown to induce significant dose-dependent inhibitory activities against different cancer cells. The determined IC₅₀ values at 48 hours of treatment (Fig. 1C) were then used for subsequent experiments. In clonogenic assay, DOE caused an irreversible damage to cancer cells because the treated cells lost their ability to form colonies (Fig. 1D and E).

3.2. DOE selectively induced autophagy and apoptosis, and not necrosis, in cancer cells

DOE induced autophagy only in HCT 116 and K562 cells with the upregulation of ATG5, LC3-II, and Beclin-1 mRNA levels, the markers of autophagy (Fig. 2A).

Morphological changes of cancer cells after treatment with DOE have demonstrated that cell damage occurred (Fig. 2B). The morphological features of apoptosis such as cell shrinkage, membrane blebbing, nuclear fragmentation, and chromatin condensation were visualized in DOE treatment followed by AO/EB staining (Fig. 2C). Hoechst staining showed that nuclei of treated cells appeared to be highly condensed or fragmented chromatin and exhibited strong blue fluorescence than control cells (Fig. 2D). The flow cytometric data of annexin V/PI staining showed that DOE induced apoptosis in cancer cells (Fig. 2E). The annexin V/PI staining indicated that the number of necrotic cells in 48 hours DOE-treated group was not statistically significant, showing that DOE favored apoptosis rather than necrosis (Table S3). The treatment of cancer cells with DOE for 48 hours resulted in fragmentation of intact DNA as evidenced by the typical ladder pattern of DNA (Fig. 2F). Compared to the control group, the numbers of TUNEL-positive cells were increased in the DOE-treated group (Fig. 2G). These data suggested that DOE had a potent cytotoxic effect with apoptosis-inducing ability on different cell lines.

3.3. DOE upregulated ROS level in cancer cells

To explore the correlation between ROS generation and apoptosis, intracellular ROS level was measured after treatment of cells with DOE, using an ROS-sensitive fluorescent dye, DCFH-DA. Increase in ROS production was observed in DOE-treated cells when compared to control cells by fluorimetric analysis and fluorescence microscopy (Fig. 3A, B). To know whether ROS plays a crucial in DOE-mediated cell death, the viability of cancer cells was measured after treatment with NAC, a reactive oxygen intermediate scavenger, followed by DOE treatment. In addition, blocking the generation of ROS by pretreatment of cells with NAC prevented the upregulation of ROS in DOE-treated cells (Fig. 3B). It was found that the presence of NAC attenuated DOE-induced cytotoxicity even after 48 hours of incubation (Fig. 3C). Taken together, these data showed that intracellular ROS plays an important role in DOE-induced apoptosis.

3.4. DOE induced both mitochondria- and receptor-mediated pathway of apoptosis

The decreased expression of pro-caspase-8, pro-caspase-9, pro-caspase-3, and pro-caspase-7 were observed in cancer cells after DOE treatment. This indicated that treatment of DOE caused activation of caspase-8, -9, -3, and -7, as evidenced by the reduction in intensity of pro-enzymes of each caspase. PARP was cleaved into the cleavage product of 89 kDa after DOE treatment (Fig. 4A). The flow cytometric analysis of caspase-3 demonstrated that DOE caused activation of caspase-3, resulting in increased expression of cleaved caspase-3 after treatment (Fig. 4B). These results together showed that DOE-induced apoptosis was mediated by caspase activation. To confirm the significance of caspase activities in DOE-induced apoptosis, cancer cells were treated with a general caspase inhibitor, z-VAD-fmk, and exposed to DOE. The MTT assay showed that either in the presence or in the absence of the caspase inhibitor, cell death occurred in DOE-treated cells (Fig. 4C). These data demonstrated that DOE-induced cell death was not attenuated by caspase blockade. Results showed that DOE increased the mRNA expression of apoptosis-inducing factor (AIF), a caspase-independent apoptosis factor in cancer cells tested (Fig. 5A).

An increase of cytochrome c protein level in cytoplasm was observed in DOE-treated cells compared to control cells (Fig. 4A), suggesting that DOE-induced apoptosis resulted in cytochrome c release from the mitochondria into the cytosol in cancer cells by activating the mitochondria-mediated pathway.

DOE significantly increased the mRNA expression of proapoptotic proteins Bax, Bim, and Bad in cancer cells and downregulated the expression of antiapoptotic proteins such as Bcl2, BclXL, and Mcl-1. DOE inhibited the expression of survivin and cIAP, two well-characterized IAPs, in cancer cells at the transcript level (Fig. 5A). Thus, DOE has demonstrated modulatory effects on the gene expression profile of Bcl2 family members.

As shown in Fig. 5B, DOE could modulate the expression of death receptors (DR4 and DR5) and ligands (Fas ligand (FasL) and tumor necrosis factor (TNF) α), resulting in the activa-
tion of caspase-8, a caspase cascade mediator of the death receptor-induced extrinsic apoptosis pathway.

3.5. **DOE exhibited ER stress in cancer cells**

Treatment of cancer cells with DOE upregulated ATF4 and CHOP mRNA expression level. As shown, the mRNA expression levels of GRP78 and GADD34 also increased in response to DOE. The XBP1 mRNA level decreased in response to DOE treatment. DOE was also found to induce splicing of XBP1 mRNA in cancer cells (Fig. 6).

![Image of Figure 2](image_url)

**Fig. 2** – DOE selectively induced autophagy and apoptosis, not necrosis, in cancer cells. Cells were treated with DOE. (A) mRNA expression of autophagy-related proteins were analyzed by RT-PCR. GAPDH was used as loading control. Each value is presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control. (B) Cancer cell morphology analysis by inverted microscopy. (C) AO/EB staining. (D) Hoechst 33342 staining observed under fluorescence microscope (magnification, ×200). (E) PI/annexin V double staining. DNA fragmentation analysis: (F) Agarose gel electrophoresis. (G) TUNEL staining. Cancer cells were incubated with DOE. DNA was isolated and analyzed by 2% agarose electrophoresis. DOE-treated cells subjected to TUNEL staining for fragmented DNA (green) by fluorescence microscopy.

AO, Acridine Orange; DOE, deoxyelephantopin; EB, ethidium bromide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.
3.6. **G2/M cell cycle arrest induced by DOE**

DOE treatment significantly increased the population of cells at G2/M phase in all cell lines (p < 0.05). DOE, after 48 hours of treatment, has caused a marked reduction of cell populations in both S phase and G1 phase (Fig. 7A, B). Because DOE induced G2/M phase cell cycle arrest, the expression of p21, p53, cyclin B1, and cdc2 was analyzed. A marked increase in p21 protein levels in cancer cells upon treatment with DOE was observed. Cyclin B1 and cdc2 levels were decreased after 48 hours of treatment with DOE. In addition, p53 level was elevated strongly after 48 hours of treatment with DOE in p53 wild-type HCT 116 and KB cells. p53 expression was also increased in DOE-treated T47D cells expressing mutant-type p53. As K562 cells lack functional p53, the expression of p53 was not observed by Western blotting (Fig. 7C).

3.7. **DOE could modulate MAPK, PI3K/Akt/m-TOR, STAT3, and β-catenin signaling pathways**

The effects of DOE on ERK1/2, SAPK/JNK, and p38 phosphorylation in cancer cells are shown in Fig. 8A. DOE treatment inhibited the phosphorylation levels of ERK1/2. By contrast, DOE treatment increased the phosphorylation levels of SAPK/JNK and p38 MAPK. No change was observed in the protein expression of ERK1/2, SAPK/JNK, and p38 MAPK.

As shown in Fig. 8A, DOE-treated cancer cells significantly decreased the pAkt protein level. No change was observed in protein expression of Akt. Furthermore, the expression of p-mTOR in the DOE-treated group was significantly lower than that in the control group.

DOE could inhibit expression of pSTAT3 in all cancer cell lines tested (Fig. 8A).

To determine whether DOE affects expression of Wnt-associated genes, mRNA levels of c-myc, β-catenin, and cyclin D1 were measured with RT-PCR. DOE could inhibit expression of the three genes. Fig. 8B summarizes the gene expression changes of c-myc, cyclin D1, and β-catenin abolished by DOE.

3.8. **Antimetastatic potential of DOE on cancer cells**

The effect of DOE on cell migration was first examined using the wound closure assay. A gradual decrease in distance between the edges of the wounded line was observed in

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Fig. 3 – Effect of DOE on the ROS production. (A) Cancer cells treated with DOE were labeled with DCFH-DA and the fluorescence was observed by microscopy. (B) Cancer cells pretreated with NAC were exposed to DOE for 48 hours, and ROS was measured fluorimetrically. (C) Cytotoxic effect of DOE with or without pretreatment of NAC in cancer cells was analyzed by MTT. Each value is presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control.

DOE, deoxylephantopin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-l-cystein; ROS, reactive oxygen species; SD, standard deviation.
the control group with increase in time (12 hours, 24 hours, 36 hours, and 48 hours). Incubation of cancer cells with DOE showed significant delay in wound closure by reducing the ability of cancer cell growth even after 48 hours of incubation (Fig. 9A). To confirm the effect of DOE on the motility of cancer cells, transwell chamber assay was performed. The number of cells that migrated to the lower chamber was reduced significantly by DOE treatment. DOE retarded the migration of cancer cells when compared with that of untreated controls ($p<0.001$; Fig. 9B). The total number of cells that invaded to the underside of the filters was significantly decreased by DOE treatment. These results show that DOE has a strong anti-invasive effect in cancer cells (Fig. 9C).

RT-PCR analysis demonstrated that DOE treatment reduced the mRNA level of matrix metalloproteinase (MMP-2), MMP-9, urokinase-type plasminogen activator (uPA), and urokinase-type plasminogen activator receptor (uPAR) in cancer cells. The tissue inhibitor of matrix metalloprotease 1 (TIMP-1) and TIMP-2 expression levels increased considerably after treatment with DOE (Fig. 9D).

3.9. **DOE abolished the expression of inflammatory genes**

A significant decrease in the mRNA expression of nuclear factor kappa B (NF-$\kappa$B) and inhibitor kappa B alpha ($\kappa\beta\alpha$) was
observed in DOE-treated cells (p < 0.01). The treatment of cancer cells with DOE completely inhibited the expression of cyclooxygenase (COX)-2 mRNA. Furthermore, DOE downregulated the mRNA expression of interleukin (IL-8) in cancer cells (Fig. 10).

4. Discussion

Cancer is a major health problem and is the second most common cause of death worldwide. Many phytochemicals are considered useful chemotherapeutic drug candidates in cancer treatment. DOE is a well-known sesquiterpene lactone isolated from E. scaber. DOE inhibited the growth of cancer cells in a time- and dose-dependent manner, and these findings are consistent with previous reports that have demonstrated the cytotoxic nature of DOE.16–20 The cytotoxic activity of a sesquiterpene lactone is attributable to the presence of \( \alpha \)-methylene-\( \gamma \)-lactone moiety in the molecule.22

The genes associated with autophagy including Beclin1, ATG, and LC3-II were not upregulated in DOE-treated T47D and KB cells, except in K562 and HCT 116 cells. To the best of our knowledge, this study is the first to demonstrate autophagy, a process of cytoplasm and cellular organelle degradation in lysosomes, in cancer cells induced by DOE.23 Autophagy and apoptosis both occur instantaneously after stress in cancer cells occasionally; at other times, only autophagy or apoptosis is observed.24 The process of death induced by DOE differs in different cancer cell lines; HCT 116 and K562 cells die via apoptosis and autophagy pathway, whereas T47D and KB cells seemed to rely mainly on apoptosis.

There is growing evidence that the increase in ROS levels in cancer cells in response to phytochemicals eventually leads to cell death.5 Treatment of DOE significantly increased the level of intracellular ROS in cancer cells. Pretreatment of NAC significantly decreased DOE-induced ROS production and prevents the cytotoxic effect of DOE. Taken together, these data showed that production of ROS is essential for DOE-induced cell death.

The two well-known apoptotic pathways are death receptor-mediated pathway (extrinsic) and mitochondria-mediated pathway (intrinsic). The Fas receptor-mediated apoptosis induced anticancer agents is well documented. The TNF-related apoptosis-inducing ligand (TRAIL) induces apoptotic cell death by binding to TRAIL death receptors DR4 and DR5. In the extrinsic pathway, caspase-8 is activated by the binding of ligands to its specific death receptors on the cell surface.25 The DOE-induced extrinsic pathway of apoptosis associated with upregulation of mRNA level of DR4, DR5, FasL,
and TNFα and caspase-8 activation in a cell type-specific manner. DOE-induced TNFα/TNF receptor mediated the apoptotic pathway without affecting Fas and FasL and activated caspase-8 in breast cancer cells, whereas FasL upregulation and t-Bid expression have been observed in DOE treated CNE cells.\(^\text{16,17}\)

The increased ratio of the proapoptotic (Bax, Bim, and Bad) to antiapoptotic (Bcl2, BclXL, and Mcl-1) Bcl2 family members would elicit cytochrome c release and activate caspase-9.\(^\text{16}\)

Modulation of Bcl2 family, caspase-9 activation, and enhanced cytochrome c level in cytoplasm suggested that mitochondrial signal pathways might be involved in DOE-induced apoptosis. In TS/A breast cancer cells, DOE did not affect the mitochondrial membrane potential, cytochrome c level, and Bcl2 family of proteins. Mitochondria-mediated apoptosis found in DOE-treated CNE cells in association with loss of mitochondrial membrane potential, translocation of cytochrome c, and alteration of Bcl2 family proteins.\(^\text{16,17}\)

Intrinsic and extrinsic apoptotic pathways activated caspase-3 followed by PARP cleavage, resulting in apoptosis.\(^\text{22}\)

DOE decreased procaspase-3, -7, -8, and -9 and increased cleaved caspase-3 and PARP protein expressions. However, it was found that the apoptosis induced by DOE was not dependent on caspase activation, because pretreatment of cancer cells with a general caspase inhibitor, z-VAD-FMK, was not affecting the cell death of cancer cells, suggesting the involvement of caspase-independent apoptotic pathways.

The mitochondrial pathway of apoptosis is mediated by the Bcl2 family of proteins that regulates passage of small molecules such as AIF through the mitochondrial transition pore. AIF is one of the apoptogenic molecules released from the mitochondria upon multiple stimuli with overexpression of AIF causing caspase-independent apoptosis.\(^\text{23}\)

During mitochondrial disruption, AIF moves to the cytosol and then to the nucleus, where it causes chromatin condensation and DNA fragmentation.\(^\text{23}\)

Overexpression of AIF mRNA levels showed that DOE-induced cell death is also linked to caspase-independent apoptosis.

Members of the IAP family (including XIAP, cIAP-1, and cIAP-2) that directly interfere with caspase-mediated apoptosis by inhibiting caspase-3, -7, and -9 are considered a valuable target to modulate apoptotic cell death in many cancer cells.\(^\text{30}\)

A decreased expression level of cIAP1/2 followed by activation of caspase-3 and -9 was observed in DOE treated cancer cells. Survivin, a member of the IAP family, increase in G2/M phases conferring resistance to apoptosis. Exposure of cancer cells to DOE decreased survivin expression at transcript level, which suggests that DOE sensitized these cancer cells to apoptosis. The apoptotic effect of DOE was exerted by the activation of both extrinsic and intrinsic pathway of apoptosis.

Chemotherapy has been reported to induce ER stress response in cancer cells. As a gatekeeper of ER stress, GRP78 activation triggers the cell apoptotic signaling pathway.\(^\text{31}\)

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**Fig. 6 – DOE-induced ER stress-related gene expression in cancer cells.** Cancer cells were treated with DOE, and XBP1, ATF4, and CHOP were evaluated by RT-PCR. GAPDH was used as a loading control. Each value is presented as mean ± SD (n = 3).

*p < 0.05, **p < 0.01, and ***p < 0.001 compared to controls.

DOE, deoxylephantopin; ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IAP, inhibitor of apoptosis; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation.
could increase GRP78 mRNA expression in cancer cells. XBP1s mRNA encodes a potent transcription factor XBP1. The XBP-1 cleaves ATF6 as well as ATF-4 to increase CHOP gene expression, triggering ER stress-specific cascade for implementation of apoptosis. CHOP regulates the transcription of Bim while suppressing the induction of Bcl2, thereby directly regulating the apoptotic machinery.\textsuperscript{32} ER stress has also been linked with mitochondria-mediated apoptosis involving the Bcl-2 family of proteins. During ER stress, calcium released from the ER is taken up by the mitochondria, causing mitochondrial inner membrane depolarization and caspase-8 activation followed by the release of cytochrome c, which activates procaspase-9, which in turn activates caspase-3, DNA fragmentation, and cell death.\textsuperscript{33} The increase of CHOP and spliced XBP1 mRNA expression and activation of Bcl2 family of proteins followed by DOE treatment indicates that DOE could induce ER stress in cancer cells.

Cell cycle is tightly regulated by cyclin, cyclin-dependent kinases (CDKs), CDK inhibitors (CDKI), and other cancer suppressor genes. DOE treatment caused an increase in G2/M phase cells with concomitant decrease in the number of G1 and S phase cells, thus confirming G2/M phase cell cycle arrest. An increase in the number of subG1 phase cells was regarded as apoptosis induction by DOE treatment. The cyclin B/cdc2 complex is required for the regulation of G2 progression and G2/M transition in all eukaryotic cells.\textsuperscript{34} P21\textsuperscript{wafl}, a CDKI, could induce G2/M cell cycle arrest by inactivating cyclin B1/cdc2 complex.\textsuperscript{35} DOE increased p21 expression in cancer cells, which was consistent with the suppressed expression of the cell cycle-related protein cyclin B1 and cdc2. In response
Fig. 8 – DOE altered the expression of MAP kinases, PI3K/Akt/mTOR, p-STAT3, and Wnt signaling pathway. (A) The cells were treated with DOE and proteins were harvested and immunoblotted with specific antibodies. β-Actin was used as a loading control. (B) The mRNA expression of β-catenin, c-myc, and cyclin D1 were analyzed by RT-PCR. GAPDH was used as a loading control. Each value is presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to controls.

DOE, deoxyelephantopin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; PI3K, phosphatidylinositol 3-kinase; p-ERK, extracellular signal-regulated kinase 1/2; p-mTOR, phosphor-mammalian target of rapamycin; p-STAT3, phospho-signal transducer and activator of transcription 3; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation.

to a variety of stresses such as DNA damage, hypoxia, and chemotherapeutic drugs, p53 protein, a key regulator of cell cycle progression and mediator of apoptosis, activates and leads to cell cycle arrest, DNA repair, and apoptosis by activation of its downstream effectors p21, GADD45, and Bax. p53 was found to be induced in the cells expressing wild type p53 (HCT 116 and KB) treated with DOE, which indicated that p53 may mediate DOE-induced apoptosis and cell cycle arrest. An increased expression of p21 and Bax, two transcriptional targets of p53, suggested that DOE treatment could modulate p53-directed cell cycle arrest and apoptosis. In T47D cells, which carry a mutant, nonfunctional form of p53, DOE caused an increase in the protein level of mutant p53 followed by apoptosis and G2/M cell cycle arrest. In response to DNA damage, p53 mutant cells undergo p53-independent cell cycle arrest and apoptosis, offering a significant therapeutic strategy for p53 mutant cancers. Upregulation of p21 protein in T47D cells treated with DOE indicated that DOE induced p53-independent p21 expression because p21 expression has played a role in cell cycle arrest. p53 protein was not detected in control and DOE-treated K562 cells, as the p53 gene was deleted. DOE caused p21 induction in K562 cells, which lacks p53. The induction of p21 is mediated by both p53 and p53-independent mechanisms and is essential for the onset of G2/M cell cycle arrest in damage response and cell senescence. Enhanced levels of both Bax and p21 proteins were also observed after DOE exposure in K562 cells, indicating that all these changes are p53-independent. These data suggested that, in addition to p53-restricted pathways, alternative pathways are involved in the DOE-induced apoptosis and cell cycle arrest.
Fig. 9 – DOE inhibited cancer cell migration and invasion and affected metastasis associated gene expression. (A) Wound closure assay. Monolayers of cancer cells were scraped and treated with DOE. Cells migrated to the wounded region were photographed (magnification, x100). (B) In vitro migration assay. Cancer cells were seeded in Boyden chamber and treated with DOE. The cells penetrated to the lower surface of the filter were stained with crystal violet, photographed (magnification, x200), and quantified. (C) In vitro migration assay. The number of DOE-treated cancer cells invaded through the gel matrix in Boyden chamber was assayed by crystal violet stain. (D) Cancer cells were treated with DOE and the expressions of genes were analyzed by RT-PCR. GAPDH was used as a loading control. Each value is presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to controls.

DOE, deoxyelephantopin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; uPA, urokinase-type plasminogen activator; uPAR, urokinase plasminogen activator receptor; SD, standard deviation; TIMP 1, tissue inhibitor of matrix metalloprotease-1.

To elucidate the molecular basis of DOE-induced apoptosis, the effect of DOE on various signaling pathways was analyzed. MAPK signaling cascades have been implicated in initiation, progression, and metastasis. The ERK pathway has been implicated as a survival factor that promote cell growth and proliferation, whereas JNK and p38 activates apoptotic signaling. DOE attenuated the expression of phospho-ERK1/2 (p-ERK1/2) and increased the expression of p-JNK and p-p38 in cancer cells without affecting their unphosphorylated forms. Moreover, it was reported that DOE inhibited ERK1/2 and activated JNK and p38 in TS/A and CNE cells.

The PI3K/Akt signaling pathway has been reported to play an essential role in various cellular processes including apoptosis, and Akt suppression could induce apoptosis through the activation of the proapoptotic factors such as Bad, GSK3β, procaspase-9, and TRAIL/APO-2L. The p-Akt phosphorylates target molecules including mTOR, which modulates cell proliferation. DOE inhibited the expression of p-Akt and p-mTOR without affecting the expression of Akt. As expected, DOE significantly increased the expression of cleaved caspase-9 and Bad in cancer cells, implying the inhibition of PI3K/Akt/mTOR signaling. The downregulation of mTOR, which prevents autophagy, was correlated with autophagy induction in cancer cells by DOE. These findings suggested that inhibition of PI3K/Akt/mTOR by DOE enhanced apoptosis in cancer cells.

The Wnt/β-catenin signaling pathway is a known regulator of cellular functions related to cancer initiation and progression. Enhanced β-catenin can act as a transcription factor in the nucleus and regulate the expression of oncogenes, including cyclin D1 and c-myc. DOE inhibited expression of β-catenin, the potential modulator of Wnt signaling, as well as its downstream targets, cyclin D1 and c-myc. These results suggested that DOE obstructs Wnt/β-catenin signalling, and inhibits cell proliferation and induce apoptosis. Activated STAT3 is frequently observed in cancer cell proliferation,
invasion, metastasis, and angiogenesis. DOE inhibited the expression of p-STAT3 in cancer cells.

The occurrence of metastasis is the major cause of mortality in cancer patients. Invasion and migration are the inherent property of malignant cells that enables them to migrate from the primary site to a secondary organ. In wound closure assay, the cells were grown rapidly in the wound to close the wound in the absence of DOE. DOE retarded the migration of cancer cells when compared with that of untreated controls. The total number of cells that invaded to the underside of the filters was significantly decreased by DOE. Binding of uPA to its receptor uPAR can activate the conversion of plasminogen to plasmin, and initiate the activation ofzymogen forms of matrix metalloproteases (MMPs) and degrade basement membrane and extracellular matrix for metastasis. TIMPs have been reported as natural MMP inhibitors that prevent the degradation of extracellular matrix by abolishing the hydrolytic activity of MMPs. DOE reduced the constitutive expression of MMP-2, MMP-9, uPA, and uPAR mRNA with a substantial increase in TIMP-1 and TIMP-2 mRNA. DOE inhibited metastasis in TS/A and A549 cells and suppressed MMP-2 and -9 expressions. Therefore, DOE could be a potential agent for the prevention of cancer metastasis.

Inflammation provides an appropriate environment for cancer progression. During chronic inflammation, proinflammatory molecules, such as cytokines, inducible nitric oxide synthase (iNOS), ROS, and NF-κB, are upregulated. ILs, TNFα, and colony-stimulating factors, secreted by cancer cells, are key molecules responsible for immune response to cancers. COX-2, the rate-limiting enzyme in prostaglandin synthesis is significantly increased in malignancies. TNFα is a proinflammatory cytokine that exerts apoptosis and promotes inflammatory processes by inducing IL-8. IL-8 is a proinflammatory cytokine secreted by many cell types, including cancer cells in response to TNF-α, and its downregulation inhibited angiogenesis and cancer growth. A significant decrease in COX-2 and IL-8 mRNA levels were observed in DOE-treated cancer cells. NF-κB is normally sequestered in the cytoplasm by interacting with IκBs. As a transcription factor, NF-κB is involved in multiple cellular processes, including cytokine gene expression, cellular adhesion, apoptosis, and metastasis, and regulate the expression of antiapoptosis genes such as TRAF, Bcl-2, cyclin D1, c-myc, and cIAP. In the present study,
DOE significantly induced apoptosis through the suppression of NF-κB and IκBα at the transcript level and suppressed NF-κB regulated gene expression. DOE suppressed breast cancer cell proliferation and induced apoptosis via inhibiting NF-κB activity by phosphorylation of IκBα followed by suppression of IKK activation. In addition, DOE also inhibited NF-κB-regulated gene expression. Our results also indicated that DOE exhibited anticancer efficacy by suppressing inflammation in cancer cells.

To conclude, DOE exerts its anticancer activities via enhancing apoptosis and autophagy; stimulating DR4, DR5, FasL, TNFα, caspase-3/7, -8, -9, PARP cleavage, cytochrome c, Bax, Bad, Bim, JNK, p38, p21, and ROS level; suppressing ERK1/2, p-STAT3, p-mTOR, p-Akt, Bcl2, BclXL, clAP, McI1, survivin, COX-2, IL-8, NF-κB, IκBα, cyclin D1, cyclin B1, cdc2, and ER stress markers; blocking cell cycle at G2/M; and preventing metastasis by modulating metastasis related genes. From these results, it could be deduced that DOE isolated from E. scaber has significant apoptotic, anticancer, and antimetastatic potential (Fig. 11).

Conflicts of interest

The authors declare no conflict of interest.

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