Ethyl gallate suppresses proliferation and invasion in human breast cancer cells via Akt-NF-κB signaling

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Abstract. Euphorbia fischeriana Steud is a traditional Chinese Medicine that is known to possess a variety of anti-carcinogenic properties. However, the bioactive constituents in Euphorbia fischeriana Steud and molecular mechanisms underlying this action in cancer treatment remain poorly understood. The present study investigated the chemotherapy activity and molecular targets of Ethyl gallate, which is identified as the major constituent extracted from the roots of Euphorbia fischeriana Steud in breast cancer cell lines in vitro. The results showed Ethyl gallate obviously decreased cell proliferation in MDA-MB-231 and MCF-7 cells in a dose- and time-dependent manner. Highly invasive MDA-MB-231 cells were found to be highly sensitive to treatment. Furthermore, significantly decreased metastatic potential of highly metastatic MDA-MB-231 cells by Ethyl gallate was identified via the inhibition of cell motility using invasion and migration through a polyethylene terephthalate membrane. Ethyl gallate treatment decreased the activity of matrix metalloproteinase-2 (MMP-2) and MMP-9 by the downregulation of mRNA levels using RT-PCR, enzymes that are critical to tumor invasion. Treatment with Ethyl gallate decreased phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor-κB (NF-κB) activation in MDA-MB-231 cells. These results indicate that Ethyl gallate suppresses proliferation and invasion in human breast cancer cells by modulating the PI3K/Akt pathway, which may contribute to inhibiting their downstream targets such as NF-κB p-65, Bcl-2/Bax, and mRNA levels of MMP-2 and MMP-9 in breast cancer cells. Thus, the present study shed new light on Ethyl gallate, an important bioactive constituent of Euphorbia fischeriana Steud, in human breast cancer treatment. The findings may provide basal theories for wide therapeutic application in human breast cancer.

Introduction

Breast cancer cases (10-15%) are characterized by an aggressive clinical with poor disease-free and overall survival and show a high rate of metastasis when compared with patients with other types of breast cancer (1-3). Currently, there is no defined standard antimetastasis treatment strategy for this disease other than traditional chemotherapy, to which it is highly resistant.

Tumor metastasis is a multistep process involving cell adhesion, cell migration and degradation of the extracellular matrix. The metastatic potential of tumor cells is influenced by a balance of the expression of proteases and their inhibitory proteins. Matrix metalloproteinases (MMPs) are a family of 24 secreted and membrane-type proteases that mediate this process. MMP-2 and MMP-9 are highly expressed in the breast and play an important role in the invasive stages of cancer (4).

The motility and invasive potential of a number of metastatic cancer cell lines has been inhibited by bioactive substances such as flavonoids (5), magnolol (6), tea catechins (7) and apigenin (8). Inhibition of MMP expression was also identified in a number of these studies.

The plant roots of Euphorbia fischeriana Steud, known as ‘lang-du’ in traditional Chinese medicine, have been used for the treatment of cancer for thousands of years (9-11). In recent years, studies have focused on Euphorbia fischeriana Steud, which has significant activity of inhibiting the growth of many tumors cell lines including leucocytoma K562, cervical carcinoma HeLa, nasopharyngeal carcinoma CNE2 and breast cancer. The bioactive substances in the roots of Euphorbia fischeriana Steud were concentrated on diterpenoids (12-15). The manner in which the bioactive constituent from Euphorbia fischeriana Steud exactly facilitates understanding of its involvement in cancer treatment remains to be determined. In the present study, five monomers from the roots of Euphorbia fischeriana Steud, and Ethyl gallate was identified as the major constituent. The aim of the study was to systematically analyze the potential activity of Ethyl gallate on cancer cell lines and its molecular targets of action. Highly invasive MDA-MB-231 and low invasive MCF-7 human breast cell lines were employed, and treated with Ethyl gallate. Cell proliferation and apoptosis were
analyzed, and the apoptotic-associated protein and expression of Bcl-2/Bax was assayed.

Highly invasive MDA-MB-231 cells were found to be highly sensitive to treatment. This observation led us to hypothesize whether the activity of Ethyl gallate extends to modulation of the metastatic potential as well. In the present study, the effect of Ethyl gallate against human highly invasive breast cancer, the abilities of cell adhesion, migration and invasion of as well as mRNA levels of MMP-2 and MMP-9 and PI3K/Akt and NF-κB signaling were evaluated. The results of the study provide important information on the role of *Euphorbia fischeriana* Steud as potential agents against the metastasis of breast cancer.

**Materials and methods**

*Preparation of Ethyl gallate.* Ethyl gallate (molecular weight of 198.1727, purity >99%), which is light brown powder, was extracted by our laboratory and dissolved in phosphate-buffered saline (PBS) and diluted in a serials concentration. The chemical structure is as shown in Fig. 1A.

*Cell lines and cell culture.* Human breast cancer cell lines, estrogen receptor-negative MDA-MB-231 cells and estrogen receptor-positive MCF-7 cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 cells were cultured in L15 medium (Gibco™ Invitrogen Corporation, Carlsbad, CA, USA), while MCF-7 cells were cultured in DMEM medium with high glucose. Both medium were supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cells were cultured at 37°C in 5% CO₂ incubator and harvested by 0.25% trypsin.

*Cell proliferation assay.* The viability of breast cancer cells treated with Ethyl gallate was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (1x10⁵/well) in 200 μl full-cultured medium were seeded in 96-well plates and incubated at 37°C. After 24-h incubation, the medium was removed and replaced with full-cultured medium containing the final concentration of Ethyl gallate at 0, 0.625, 1.25, 2.5, 5, 10, 20, 40 and 80 μg/ml. All groups were quadrupled. At 12, 24 and 48 h after treatment with Ethyl gallate, MTT solution was added to each well (500 μg/ml final concentrations) and incubated at 37°C for 4 h. The supernatant was removed, and 150 μl of dimethyl sulfoxide (DMSO) was added. The optical density value of the solution was read at 570 nm using a spectrophotometer (Safire2; Tecan Group Ltd., Maennedorf, Switzerland). The percentage of cell viability was determined by comparing the cell density of the drug-treated cells with that of untreated controls.

*Analysis of apoptosis.* MDA-MB-231 cells were treated with Ethyl gallate at a concentration of 0, 2.5, 5.0 and 10.0 μg/ml for 24 h. Annexin V assays were carried out using the Annexin V-FITC apoptosis detection kit (Becton-Dickinson, San Jose, CA, USA). The treated cells were washed in cold PBS, and then resuspended gently in 400 μl of binding buffer prior to the addition of Annexin V-FITC and propidium iodide (PI). The cells were incubated for 15 min at room temperature, and 10,000 cells were analyzed for apoptosis using flow cytometry (BD Biosciences, San Jose, CA, USA). The percentage of apoptotic cells was quantified using CellQuest software (Becton-Dickinson). The experiments were repeated in triplicate separately.

*Matrigel adhesion assay.* The 96-well plates were precoated with 25 μg/well of Matrigel (BD Biosciences). The MDA-MB-231 cells (1x10⁵/well) were incubated in 1 ml of serum-free medium containing Ethyl gallate at concentrations of 0, 2.5, 5.0 and 10.0 μg/ml and labeled by derivatives of indocarboxyline (DiI; 3 μg/ml) for 2 h at 37°C, respectively. Then cells were washed twice with PBS and seeded into the precoated wells and incubated for 30 min at 37°C in a 5% CO₂ incubator. The cells were washed twice with PBS gently to remove unadhered cells, and the number of cells adhered to the Matrigel was counted in five randomly selected microscopic fields per well and photographed by fluorescence microscope. Independent experiments were performed at least three times.

*In vitro migration and invasion assay.* Tumor cell migration and invasion were examined using Transwell chambers (Costar, Corning, Inc., Corning, NY, USA). Briefly, Matrigel was coated the upper chamber for invasion (not for migration). MDA-MB-231 cells (5x10⁴ for invasion and 2x10⁵ for migration) were incubated in 1 ml of serum-free medium containing Ethyl gallate at concentrations of 0, 2.5, 5.0 and 10.0 μg/ml for 2 h, respectively. washed in PBS and seeded in the upper chamber in 200 μl of serum-free medium, while the lower chamber was filled with 600 μl of medium supplemented with 10% of FBS. After 24-h incubation at 37°C, the cells on the upper chambers were scraped with a cotton swab. Cells migrating or invading to the lower surface were fixed with methanol for 30 min, stained with 0.1% crystal violet for 20 min, washed with PBS, photographed with fluorescence microscope and incorporated dye was dissolved in 10% acetic acid. The optical densities of each well were measured using a microplate reader at 570 nm. Experiments were repeated three times.

*Protein extraction and western blot analysis.* Cells were treated as described for the apoptosis assay. Total cellular proteins were extracted. Equivalent amounts of cellular protein were electrophoresed in 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) and blocked in 5% non-fat milk at 4°C overnight. The membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody in 5% non-fat milk for 1 h at room temperature. The bands were detected by enhanced chemiluminescence techniques (Amersham Life Science, Piscataway, NJ, USA) and GAPDH in 5% non-fat milk at 4°C overnight. The membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody in 5% non-fat milk for 1 h at room temperature. Band densities were quantified by using BandScan software.

*Semi-quantitative reverse transcription-PCR.* Total cellular RNA was extracted using TRIzol reagent (Invitrogen) and quantified by spectrophotometry. RT-PCR reaction was carried out using an RT-PCR kit (Takara Biotechnology, San Jose, CA, USA). The results of the study provide important information on the role of Ethyl gallate as potential agents against the metastasis of breast cancer.
Dalian, China) according to the manufacturer’s instructions. PCR amplification was carried out with the following primers: MMP-2, forward: 5’-GGATGATGCCTTTGCTCG-3’ and reverse: 5’-CAGTGGACATGGCGGTCT-3’; MMP-9, forward: 5’-TCCCTGGAGACCTGAGAACC-3’ and reverse: 5’-GGC AAGTCTTCCGAGTAGTTT-3’; β-actin, forward: 5’-ATCATGGTTTGTTCATCCTTCAACACC-3’ and reverse: 5’-GGC AAGTCTTCCGAGTAGTTT-3’; β-actin, forward: 5’-ATCATG TTTGAGACCTTCAACACC-3’ and reverse: 5’-GGC AAGTCTTCCGAGTAGTTT-3’; β-actin, forward: 5’-ATCATG TTTGAGACCTTCAACACC-3’ and reverse: 5’-GGC AAGTCTTCCGAGTAGTTT-3’. The amplification products were separated through a 1.5% agarose gel electrophoresis. The intensity of each band was quantified using Scion Image software (Scion Corporation, Frederick, MD, USA). Results for each detected band intensity were normalized to β-actin band intensity values.

Statistical analysis. Values were expressed as means ± standard deviation and analyzed by the SPSS 13.0 software to evaluate the statistical difference. One-way analysis of variance (ANOVA) was used to establish whether significant differences existed among multiple groups. P<0.05 was considered to indicate a statistically significant result.

Results

Ethyl gallate inhibits proliferation and induces apoptosis of breast cancer cells in a dose- and time-dependent manner. The effects of Ethyl gallate on the proliferation of MDA-MB-231 [estrogen receptor-negative (ER-)] and MCF-7 [estrogen receptor-positive (ER+)] human breast cancer cell lines were investigated using an MTT assay. The same amount of cells was seeded and treated with a series of concentrations of Ethyl gallate from 0 to 80 µg/ml. The results showed that Ethyl gallate significantly inhibited cell proliferation in the MDA-MB-231 and MCF-7 cells in a dose- and time-dependent manner following treatment for 12, 24 and 48 h. The IC50 values at 12, 24 and 48 h were 17.85, 6.55 and 2.95 µg/ml and 32.53, 16.88 and 9.40 µg/ml for MDA-MB-231 (Fig. 1B) MCF-7 (Fig. 1C) cells, respectively. However, MDA-MB-231 cells (ER-) were more sensitive to Ethyl gallate than MCF-7 cells (ER+).

To determine whether the decreased cell numbers occurred due to the induction of cell death, apoptosis was quantitatively analyzed by flow cytometry. Following treatment with Ethyl gallate at concentrations of 2.5, 5.0 and 10.0 µg/ml for 24 h in the MDA-MB-231 cells, the percentage of apoptosis was 21.50, 33.86 and 39.77%, respectively (Fig. 1D). The results suggested that apoptosis is an important mechanism of Ethyl gallate in the induction of breast cancer cell death.

Effect of Ethyl gallate on Bcl-2 and Bax proteins involved in apoptosis. Pro-apoptotic molecular mechanisms underlying Ethyl gallate were examined. We investigated the expression of Bcl-2 and Bax, which are pivotal regulators of cell growth and apoptosis. Western blot analysis revealed that Ethyl gallate decreased Bcl-2 but increased Bax in a dose-dependent manner (Fig. 2). Thus, Ethyl gallate may induce apoptosis by altering the Bax/Bcl-2 ratio favoring apoptosis.

Ethyl gallate reduces cell adhesion, migration and invasion. Considering that highly invasive breast cancer cells were more sensitive to treatment with Ethyl gallate than poorly invasive ones, we determined whether Ethyl gallate inhibited the invasive behavior of breast cancer cells. Tumor invasion involves cell adhesion to the extracellular matrix, migration and invasion to Matrigel. To investigate the effect of Ethyl gallate on breast cancer invasion potential, cell adhesion, migration and invasion assays were examined in highly invasive MDA-MB-231...
cells. As shown, the number of cells that adhered to Matrigel was 143.78±8.53, 121.38±6.38, 76.34±6.32 and 42.0±5.18 respectively (Fig. 3A). Twenty-four hours after cell migration and invasion, the optical density of MDA-MB-231 cells were 0.623±0.053, 0.476±0.064, 0.536±0.059, 0.385±0.043, 0.284±0.048, 0.225±0.016, 0.027±0.006 and 0.014±0.002 respectively, following cell treatment with Ethyl gallate at concentrations of 2.5, 5.0 and 10.0 µg/ml for 2 h (Fig. 3B).
The results suggested that Ethyl gallate at short effect time significantly suppressed the adhesion, migration and invasion of MDA-MB-231 cells with the inhibition rate of 13.40, 13.89, 19.12, 46.90, 54.42, 52.74, and 70.79, 95.67, 97.06% (Fig. 3C).

**Inhibition of MMP-2 and MMP-9 expression by Ethyl gallate.**
Degradation of the extracellular matrix relative proteins, MMP-2 and MMP-9 is important in the invasive stages of cancer. RT-PCR detection demonstrated that Ethyl gallate at 5.0 and 10.0 µg/ml significantly reduced the mRNA levels of MMP-2 (Fig. 4A) and MMP-9 (Fig. 4B) in MDA-MB-231 cells following cell treatment for 24 h with Ethyl gallate.

**Inhibition of cell signaling pathway by Ethyl gallate.** The importance of the PI3K/Akt and NF-κB signaling pathways to tumor cell growth and invasion was investigated. NF-κB is a critical transcription factor involved in the regulation of MMP-9 expression. To further understand the inhibitory mechanisms of Ethyl gallate on MMP-9 transcriptional regulation, the PI3K/Akt and NF-κB signaling pathways were investigated by western blot analysis. The results revealed that Ethyl gallate significantly reduced Akt phosphorylation at 5.0 and 10.0 µg/ml (P<0.05 and 0.01; Fig. 5A), and also decreased the activity of NF-κB at 5.0 µg/ml (P<0.05) and 10.0 µg/ml (P<0.01; Fig. 5B). By contrast, the inhibition of PI3K/Akt
leading to downregulation of NF-κB activation may be one mechanism of Ethyl gallate against MDA-MB-231 cell proliferation and invasion.

Discussion

Euphorbia fischeriana Steud is known to inhibit tumor cell growth. The bioactive constituents from the roots of Euphorbia fischeriana Steud were concentrated on diterpenoids. However, in the present study, we found that Ethyl gallate, which is identified as the major constituent in Euphorbia fischeriana Steud extracts, potently inhibits proliferation and induces apoptosis of breast cancer cells in dose- and time-dependent manners in vitro. MDA-MB-231 cells (ER+) were more sensitive to Ethyl gallate than MCF-7 cells (ER-) (Fig. 1). This result shows that Ethyl gallate is an important bioactive constituent in the chemotherapy of breast cancer through the pathways of marked induction of cell apoptosis. Induction of apoptosis was primarily due to altering Bax/Bcl-2 ratio (Fig. 2), providing evidence for understanding the manner in which Euphorbia fischeriana Steud functions in cancer treatment.

Metastasis is the main cause of mortality in cancer patients (16). During the complicated processes of metastasis, the adhesion to the extracellular matrix (ECM), migration and invasion of cancer cells are the most pivotal steps for motility to distant sites. Many bioactive substances have been proven to inhibit an invasive potential of metastatic cancer cell lines, including prostate, lung and breast (17-19). In the present study, we have shown that Ethyl gallate markedly inhibited cell adhesion in Matrigel (Fig. 3) and the migratory abilities of highly invasive MDA-MB-231 breast cells. The anti-invasive capacity of Ethyl gallate may be important in decreasing mortality and improving the survival time in ER breast cancer patients.

For tumor invasion, matrix metalloproteinases (MMPs) are the best documented critical proteolytic enzymes associated with degradation of ECM (20-22). It is believed that this characteristic initiates the metastatic process and enables tumor cells to invade and spread to various secondary sites. To facilitate metastasis, tumor cells depend on the activity of more than one MMP, enabling them to cross the tissue barriers they encounter during the process of invasion. Although many MMPs have been identified, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the enzymes most pivotal to degrading ECM (23,24), and they are highly expressed in highly metastatic tumors (25). MMP activity is regulated by gene transcription, while its expression level is directly associated with invasion and metastasis of tumor. Thus, MMP-2 and MMP-9 are considered to be a target for breast cancer therapy by suppressing breast cancer invasion (26). The present study demonstrated that Ethyl gallate treatment significantly inhibited the activity of MMP-2 and MMP-9 by downregulating mRNA expression levels in MDA-MB-231 cells compared with the untreated control (Fig. 4). Therefore, MMP-2 and MMP-9 may be Ethyl gallate-responsive mediators whose degradation of ECM may cause subsequent cancer migration and invasion.

In addition, the efficacy of Ethyl gallate can be explained by interference with the PI3K/Akt and NF-κB signaling pathways, which have been found to be important in growth, invasion and metastasis of tumor cells (27). It has been shown that the NF-κB signal a major pathway for modulating MMP-9 expression (28,29). In the present study, Akt phosphorylation and NF-κB activation were found to be inhibited by Ethyl gallate treatment, although there was no effect on total Akt (Fig. 5). NF-κB is a nuclear transcription regulator for Bcl-2 transcription (30,31). The Bcl-2, Bax, p-Akt and NF-κB have become the main targets of action by anticancer agents (32-34). Ethyl gallate inhibits the PI3K/Akt and NF-κB/Bcl-2 signaling pathways by suppressing the phosphorylation of Akt and NF-κB expression in MDA-MB-231 cells. This inhibition may contribute to downregulation of the Bcl-2/Bax ratio and mRNA expression levels of MMP-2 and MMP-9 in human breast cancer cells. These data provide a basic mechanism for Ethyl gallate chemotherapeutic properties of human breast cancer cells.

Abnormal growth and metastasis are considered as important biological properties of cancer cells. An agent that efficiently inhibited these biological properties of cancer cells is a potential candidate for the suppression of cancer progression. Our data suggest that Ethyl gallate is an effective agent to target breast cancer proliferation as well as migration and invasion. These inhibitory effects are at least partially mediated by interference with the Akt-NF-κB signaling pathway.

Future studies are required to analyze the precise mechanism(s) of Ethyl gallate and to exploit its full potential for breast cancer chemotherapy. In vivo studies are pivotal to confirm these in vitro mechanisms and determine future therapeutic applications of Ethyl gallate against breast cancer.

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References


