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Original Article

Osthole inhibits proliferation of human breast cancer cells by inducing cell cycle arrest and apoptosis

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Abstract

Recent studies have revealed that osthole, an active constituent isolated from the fruit of *Cnidium monnieri (L.) Cusson*, a traditional Chinese medicine, possesses anticancer activity. However, its effect on breast cancer cells so far has not been elucidated clearly. In the present study, we evaluated the effects of osthole on the proliferation, cell cycle and apoptosis of human breast cancer cells MDA-MB 435. We demonstrated that osthole is effective in inhibiting the proliferation of MDA-MB 435 cells, The mitochondrion-mediated apoptotic pathway was involved in apoptosis induced by osthole, as indicated by activation of caspase-9 and caspase-3 followed by PARP degradation. The mechanism underlying its effect on the induction of G1 phase arrest was due to the up-regulation of p53 and p21 and down-regulation of Cdk2 and cyclin D1 expression. Were observed taken together, these findings suggest that the anticancer efficacy of osthole is mediated via induction of cell cycle arrest and apoptosis in human breast cancer cells and osthole may be a potential chemotherapeutic agent against human breast cancer.

Keywords: osthole, breast cancer, proliferation, cell cycle, apoptosis

Introduction

Breast cancer is an important public health problem worldwide and continues to rank as one of the top causes of death in women[1]. Despite diagnostic and therapeutic advances the long-term prognosis of breast cancer remains poor. Recently, more attention has been paid to the natural products in the treatment of breast cancer because they are associated with low toxicity. For example, genistein, isoliquiritigenin and resveratrol have been reported to be potentially excellent candidates for use as chemopreventive agents against estrogen receptor (ER) positive breast cancer[2-4]. However, ER-negative breast cancers are also more aggressive and have poorer clinical outcomes than ER-positive cancers[5]. Therefore, identifying novel drugs for the treatment of ER negative breast cancer is urgently required.

Osthole (7-methoxy-8-isopentenoxycoumarin) is a natural coumarin and bioactive compound isolated from the fruit of *Cnidium monnieri (L.) Cusson*. It has been reported that osthole possesses vasorelaxant[6], anti-osteoporotic[7] and anti-inflammatory effects[8,9].

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Recently, osthole has received more attention for its anti-tumor activity[10–13]. However, the effects of osthole on breast cancer cells and the underlying mechanisms are poorly understood.

In the present study, we examined the effects of osthole on the proliferation, cell cycle and apoptosis of MDA-MB 435 cells, which are ER-negative and highly metastatic human breast cancer cells.

Materials and methods

Reagents

Osthole was obtained from Jiangsu Institute for Food and Drug Control (Nanjing, Jiangsu, China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for p53 and p21, cyclin D1, Cdk2, Bax, Bcl-2, pro-caspase-9 and pro-caspase-3 were from Bioworld Technology (West Palm Beach, USA). Antibody for PARP was from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody and anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

MDA-MB 435 cells were cultured in Dulbecco´s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin. Cultured cells were incubated at 37°C in a humid chamber at 5% CO_2.

MTT assay

MDA-MB 435 cells at the logarithmic growth phase were collected and seeded in 96-well tissue culture plates (8×10^3 cells/well) and allowed to adhere overnight. After the cells were treated with osthole (0, 20, 40, 60, 80 or 100 mol/L) for 24, 36 or 48 hours, 20 μL of MTT (5 mg/mL) was added into each well and the cells were incubated at 37°C for 4 hour. followed by 150 μL of dimethyl sulfoxide (DMSO) and incubated for 1 hour at 37°C. The absorbance at 570 nm was measured using a Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Flow cytometry

After treatment with osthole, MDA-MB 435 cells were resuspended in 70% cold ethanol and incubated on ice for at least 1 hour. Cells were resuspended in FACS buffer (0.5 mg/mL RNase A, 0.38 mmol/L sodium citrate and 0.15 mg/mL propidium iodide) and incubated for 30 minutes at 37°C in the dark. Cell cycle profile was analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

To estimate the number of apoptotic cells, MDA-MB 435 cells treated with osthole were harvested and centrifuged at 1000 rpm for 10 minutes, and washed with PBS. Then the cells were fluorescently labeled with 20 μL of 1×binding buffer (10 mmol/L Hepes/NaOH, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, pH 7.4), 5 μL of annexin V-FITC and 5 μL of PI. After incubation at room temperature for 15 minutes in dark, the cells were analyzed immediately by FACScan flow cytometer.

Western blotting assays

MDA-MB 435 cells were treated with osthole (0, 25, 50 or 100 μmol/L) for 36 hours. Cells were washed with PBS and lysed with RIPA buffer (50 mmol/L Tris, pH7.4, 0.5% NP-40 and 0.01% SDS)) plus protease inhibitors (Roche). Total protein (30 μg) was boiled for 5 minutes in 1×loading buffer, chilled on ice and then separated on 10% sodium dodecyl sulfate-polyacrylamide gels and electrobloted onto polyvinylidene fluoride membranes (Millipore, USA). Non-specific protein interactions were blocked by incubation with 5% nonfat milk in TBST buffer (50 mmol/LTris-HCl, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.6) at 4°C for 1 hour. The membranes were incubated overnight with the primary antibodies in the blocking buffer at 4°C. Unbound antibody was removed by washes in TBST buffer for three times (10 minutes/time). Membranes were then incubated with horseradish peroxide-conjugated secondary antibody for 1 hour at room temperature followed by washes with TBST buffer for three times (10 minutes/time). The blots were developed with ECL reagent (Applygen).

Statistical analysis

All of the experiments were independently repeated at least three times. Comparisons between control and treatment groups were analyzed by one-way ANOVA and Tukey´s post-hoc test or Student´s *t* test. A probability value of $P < 0.05$ was considered statistically significant.

Results

Osthole inhibited the proliferation of MDA-MB 435 cells

To determine whether osthole has proliferation inhibitory effects on breast cancer cells, MDA-MB 435 cells were treated with different concentrations of osthole (0, 20, 40, 60, 80 and 100 μmol/L) for 24, 36 and 48 hours, respectively. Cellular proliferation was evaluated by MTT assay. As shown in ***Fig.1***, the

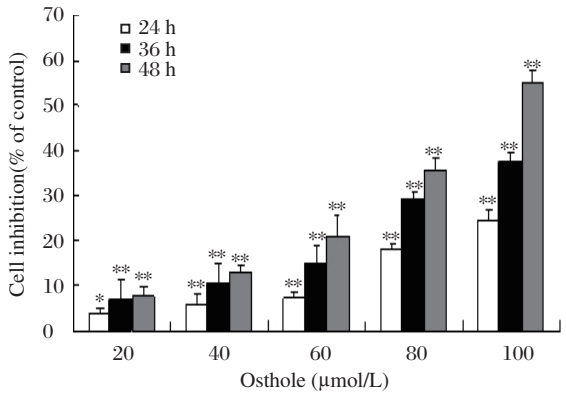

Fig 1. **Effects of osthole on the proliferation of MDA-MB 435 cells.** $^{*}P < 0.05$, $^{**}P < 0.01$ **vs. control (0 μ mol/L osthole)**

proliferation of MDA-MB 435 cells was inhibited by osthole in a dose- and time-dependent manner. When MDA-MB 435 cells were treated with osthole (0, 20, 40, 60, 80 and 100 μmol/L) for 48 hours, the growth inhibitory rate was (7.8±2.2)%, (13.0±1.2)%, (21.0±4.5)%, (35.6±2.5)% and (54.8±2.8)%, respectively. When the cells were treated with 100 μmol/L of osthole for 24, 36 and 48 hours, the growth inhibitory rate was (24.6±1.9)%, 37.2±2.4)% and (54.8±1.2)%, respectively.

Osthole induced G1/S arrest of MDA-MB 435 cells

To elucidate whether the proliferation inhibitory effect of osthole was due to cell cycle arrest, MDA-MB 435 cells were treated with osthole (0, 25, 50 and 100 μmol/L) for 36 hours and stained with propidium iodide for flow cytometry analysis. As shown in ***Fig. 2***and ***Table 1***, the G1 phase cell population of osthole-treated cells increased significantly in a dose-dependent manner. Treatment with 100 μmol/L of osthole led to G1 phase accumulation by 72.8% , which was accompanied by a corresponding reduction in the percentages of cells in S phase ***(Fig. 2*** and ***Table 1)***. Moreover, 100 μmol/L osthole significantly increased the sub-G1, suggesting that inducing apoptosis may be also population involved in osthole mediated inhibition of the proliferation of MDA-MB 435 cells.

Osthole induced apoptosis of MDA-MB 435 cells

To determine whether cells treated with osthole underwent apoptosis in addition to cell cycle arrest, MDA-MB 435 cells were treated with osthole and stained with annexin V and propidium iodide for flow cytometry analysis. As shown in ***Fig. 3***, the number of apoptotic cells was increased compared with control. When MDA-MB 435 cells were treated with 100 μM of osthole for 36 hours, the proportion of the apoptotic cells was over three times higher than the control.

Effects of osthole on cell cycle control proteins and apoptosis–associated proteins

As shown in ***Fig. 4***, the expression of p53 and p21 were obviously increased, and the expression of Cdk2 and cyclin D1 were significantly decreased after osthole treatment. To explore the mechanism by which osthole induced apoptosis, we analyzed the levels of pro-caspase-3 and PARP by Western blotting analysis. As shown in ***Fig. 5***, pro-caspase-3 in MDA-MB 435 cells was reduced in a dose-dependent manner after treatment with osthole. In addition, full-length PARP was decreased progressively, Caspase-9 is an important intracellular amplifier of caspase signaling downstream of mitochondria. To further define whether osthole induces apoptosis via the intrinsic pathway, the level of pro-caspase-9 was examined. As shown in ***Fig. 5***, the level of pro-caspase-9 in MDA-MB 435 cells was reduced in a dose-dependent manner after treat–

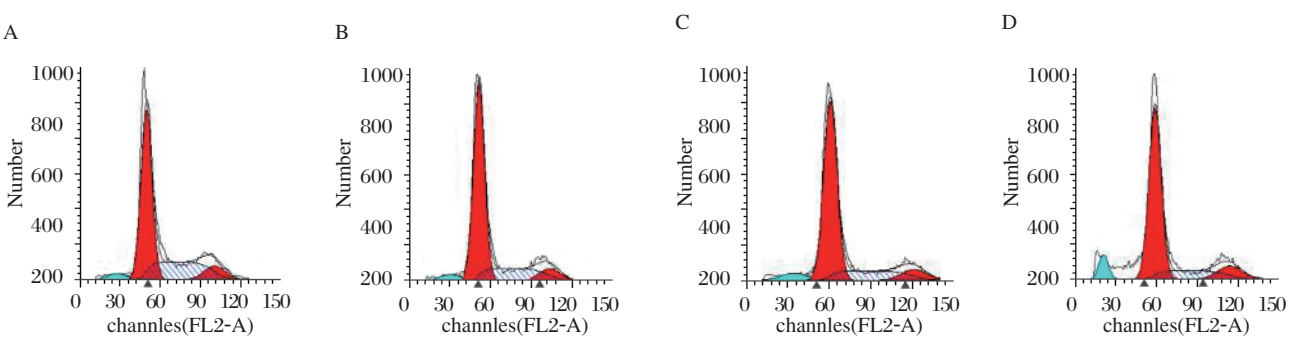

Fig 2. **Effects of osthole on the cell cycle distribution of MDA-MB 435 cells.** MDA-MB 435 cells were treated with osthole for 36 hours. Cells were harvested and prepared for the cell cycle detection using flow cytometer as described in the Materials and Methods. Images are shown for one of three separate experiments. A: Control group. B: osthole 25 μmol/L. C: osthole 50 μmol/L. D: osthole 100 μmol/L.

Table 1 **Cell cycle distribution (%) of MDA-MB 435 cells after osthole treatment.**

	Control	25 (μmol/L)	50 (μmol/L)	100 (μmol/L)
G1	59.63±0.22	67.95±1.13	71.94±0.29	72.81±0.78
S	30.13±0.075	22.44±0.49	18.85±0.395	17.14±0.44
G2/M	10.25±0.295	9.62±0.63	9.22±0.1	10.06±1.22

ment with osthole.

Osthole upregulated Bax and downregulated Bcl-2 expression

The mitochondrial intrinsic pathway of apoptosis is regulated by the balance between the pro- and anti apoptotic Bcl-2 family of proteins[14–16]. We performed immunoblot analysis to determine whether the apoptosis induced by osthole was associated with changes of antiapoptotic (Bcl-2) and proapoptotic (Bax) proteins. As shown in ***Fig. 6***, the expression of Bax was increased and the expression of Bcl-2 was decreased following osthole treatment. These results suggest that osthole induces apoptosis by suppression of anti-apoptotic protein expression and promotion of pro-apoptotic protein expression.

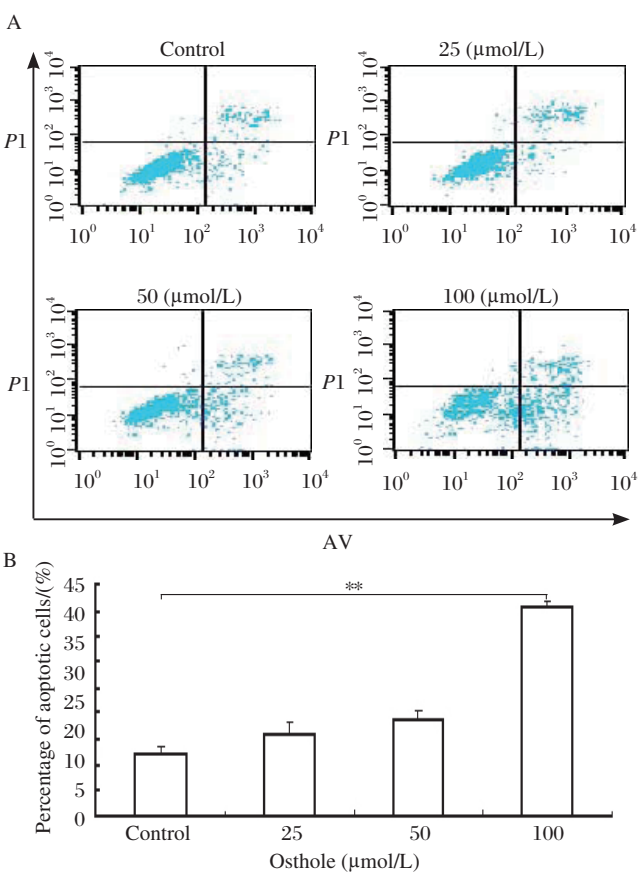

Fig 3. **Effects of osthole on the apoptosis of MDA-MB 435 cells.** MDA-MB 435 cells were treated with osthole (0, 25, 50 and 100 μmol/L) for 36 hours. Cells were harvested and analyzed with annexin V and propidium iodide using flow cytometer as described in the Methods. A: Representative histograms of three separate experiments. B: Results of three separate experiments are shown. Values are expressed as mean±SD. $^{**}P < 0.01$.

Discussion

Natural products have always been important resources either as therapeutic agents or as lead compounds for the production of pharmaceutical compounds[17]. Because of its significant and diverse pharmacological activities, including anticancer, antisteoporotic, and antiproliferative, osthole has recently been considered as a very promising natural lead compound for new drug discovery. The anticancer effect of osthole has been reported in a few studies[18-22]. It is reported recently that osthole is able to inhibit the migration and invasion of breast cancer cells[20]. In the present study, we demonstrated that the possible roles of osthole on MDA-MB 435 cells, an estrogen receptor negative and highly metastatic human breast cancer cell line, were 1) to inhibit the proliferation of cells in a dose- and time-dependent manner, 2) to arrest the cell cycle at G1 phase via the up-regulation of p53 and p21 as well as down-regulation of cyclin D1 and Cdk2, and 3) to induce apoptosis via activation of caspase-9 and caspase-3 followed by cleavage of PARP.

In the proliferation assay, we clearly demonstrated that osthole inhibited the proliferation of MDA-MB 435 cells in a dose- and time- dependent manner ***(Fig. 2)***. The inhibition rate was 54.8% when the cells were treated with 100 μmol/L of osthole for 48 hours. These results indicate that osthole may have significant anti-cancer effect on human breast cancer cells. To characterize the underlying mechanisms by which osthole inhibits the proliferation of MDA-MB 435 cells, we tested whether osthole had the capacity to block cell cycle progression by flow cytometric analysis. The results showed that upon treatment with 100 μM of osthole for 36 hours, the proportion of cells in G1 phase increased to 72.8%, being remarkablely higher than that of the control. Therefore, G1 phase arrest may be one of the causes which lead to the inhibition of MDA-MB 435 cell proliferation by osthole. Our results of flow cytometry analyses also showed that osthole markedly induce apoptosis of MDA-MB 435 cells, suggesting that inducing apoptosis may also contribute to the anti-proliferative function of osthole against breast cancer cells.

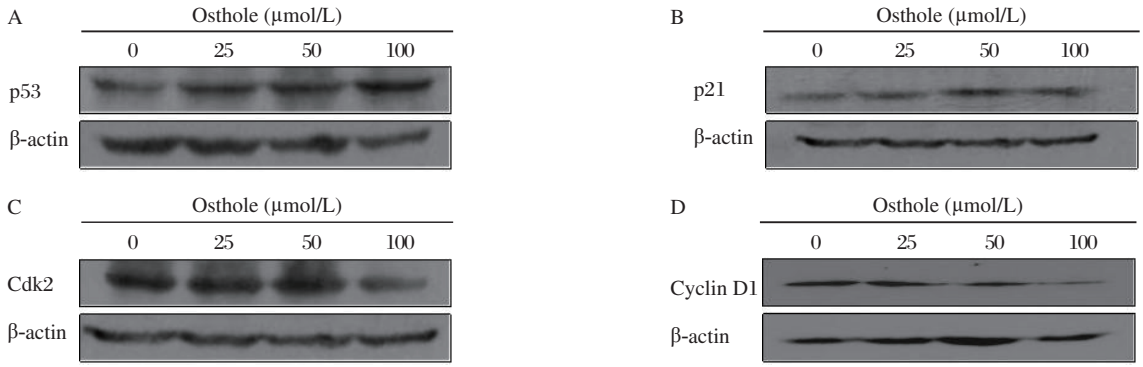

Fig 4. **Effects of osthole on the expression of p53 and p21 as well as Cdk2 and cyclin D1.** MDA-MB 435 cells were treated with osthole (0, 25, 50 and 100 μmol/L) for 36 hours. Cell lysates were immunoblotted with anti-p53 (A) anti-p21 (B) anti-Cdk2 (C) and anti-cyclin D1 (D) antibodies, respectively. β-actin served as loading control.

Cell cycle control is a highly regulated process that involves a complex cascade of events. Modulation of the expression and function of the cell cycle regulatory proteins including Cdks and cyclins provides an important mechanism for the inhibition of growth[18–20]. To understand the mechanism by which osthole inducing G1 phase arrest, Cdk2 and cyclin D1 which are key mediators involved in controlling G1 phase progression and G1/S phase transition. The results showed that treatment with osthole led to down-regulation of Cdk2 and cyclin D1. In addition to the binding of cyclins, Cdk activity is modulated by positive and negative interfering factors. Among these factors, p21 is one of the most interesting inhibitors, since it has multiple binding capacities not only for CDK[21,22] but also for cyclins[23]. our results showed that p21 was up-regulated after MDA-MB 435 cells were treated with osthole. The p53, as a tumor suppressor protein, governs the long-term cellular response to genotoxic stress by inducing a p21-dependent cell cycle arrest or apoptosis[24, 25]. Furthermore, we examined the expression of p53 and found that p53 was up-regulated after osthole treatment. These data suggest that G1 phase arrest induced by osthole was associated with the up-regulation of p53, p21 and down-regulation of Cdk2 and cyclin D1. However, it was reported that osthole inhibited the growth of human lung cancer A549 cells by inducing G2/M arrest[13], indicating that the mechanism by which osthole induces cell cycle arrest may be cell type-dependent.

Apoptosis plays important roles in the development, homeostasis of cell populations and pathogenesis of diseases[26]. It is mediated either through death receptors (extrinsic pathway) or through the mitochondrial pathway (intrinsic pathway)[27-29]. Both extrinsic pathway and intrinsic pathway involve the activation of members of the caspase family of cysteine proteases in a hierarchical cascade, with caspases functioning as triggers and executioners of the apoptotic process. Activation of caspase-9 is involved in the intrinsic pathway, whereas activation of caspase-8 is involved in the extrinsic pathway. Caspase-3 is a major executioner protease, responsible for initiating the apoptotic program and it may be activated either by caspase-8 or by caspase-9[30, 31]. Cleavage of PARP, which is a well-known substrate for caspase-3 in the apoptotic events, facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis[14]. Activation of caspase-9 and caspase-3 is usually regarded as a hallmark of mitochondrial cell death[32]. To explore the possible mechanisms of osthole induced apoptosis, we examined the levels of pro-caspase-9, pro-caspase-3 and PARP by Western blotting. The results showed that treatment with osthole decreased pro-caspase-9, pro-

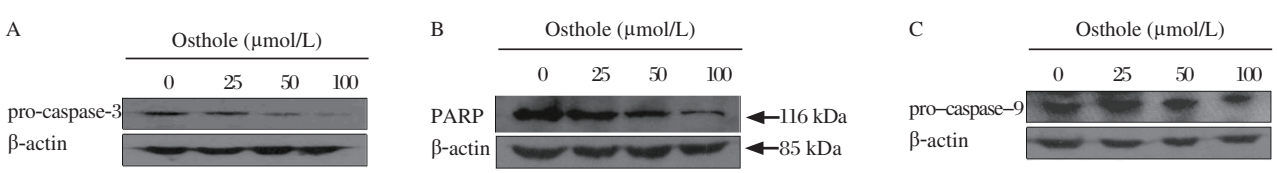

Fig 5. **Effects of osthole on the levels of pro-caspase-3, PARP and pro-caspase-9.** MDA-MB 435 cells were treated with osthole (0, 25, 50 and 100 μmol/L) for 36 hours. Cell lysates were immunoblotted with anti-pro-caspase-3 (A) anti-PARP (B) and anti-pro-caspase-9 (C) antibodies, respectively. β-actin served as loading control.

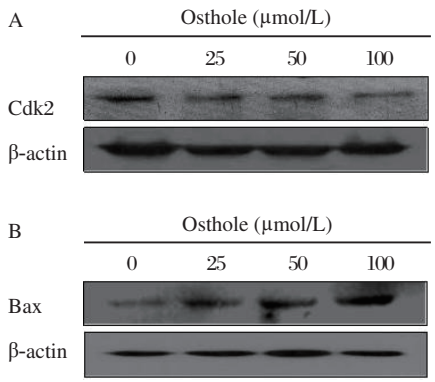

Fig 6. **Effects of osthole on the levels of Bcl–2 (A) and Bax (B).** MDA-MB 435 cells were treated with osthole (0, 25, 50 and 100 μmol/L) for 36 hours. Cell lysates were immunoblotted with anti-Bcl-2 and anti-Bax antibodies, respectively. β-actin served as loading control.

caspase-3 and caused specific cleavage of the caspase-3 substrate PARP, indicating that the mitochondrial signaling pathway may be involved in osthole induced apoptosis. Our results of Bcl-2/Bax examination are also consistent with the data that osthole induces apoptosis by down-regulating the expressions of Bcl-2 and up-regulating the expressions of Bax in human lung cancer A549 cells[13].

In summary, our study demonstrated that osthole is effective in inhibiting cell proliferation, and inducing G1 phase arrest and apoptosis in breast cancer cells MDA-MB 435. G1 phase arrest induced by osthole was associated with the up-regulation of p53, p21 and down-regulation of Cdk2, and cyclin D1. Treatment with osthole led to the activation of caspase 9 and caspase 3 followed by PARP degradation, suggesting that the mitochondrion-mediated apoptotic pathway may be involved in the mechanism of apoptosis induced by osthole. Taken together, these results suggest that osthole may be a potential candidate as chemotherapeutic agent against human breast cancer.

Acknowledgement

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