

# Phloretin induces apoptosis of human esophageal cancer via a mitochondria-dependent pathway

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**Abstract.** 2,4,6-trihydroxy-3-(4-hydroxyphenyl)-propionophenone (phloretin) is found in apple tree leaves and the Manchurian apricot, and is a potent compound that exhibits anti-inflammatory, antioxidant and antitumor activities. However, the effect of phloretin on esophageal cancer cells is not well-defined. The present study aimed to examine whether and how phloretin induced apoptosis in human esophageal cancer cells. EC-109 cells were cultured in Dulbecco's modified Eagle's medium and incubated with 60, 70, 80, 90 and 100  $\mu$ g/ml phloretin for 6, 12, 24 and 48 h. Cell proliferation was measured by an MTT assay. Cell apoptosis rate was measured using flow cytometric analysis subsequent to propidium iodide (PI) staining. The protein expression levels were determined by western blot analysis. It was found that phloretin significantly decreased viable cell numbers in a dose- and time-dependent manner and induced apoptosis in EC-109 cells. Additionally, phloretin exhibited potent anticancer activity *in vitro*, as evidenced by the downregulation of the anti-apoptosis-associated molecule B-cell lymphoma 2 (bcl-2) and an increase in the levels of the apoptosis-associated molecules bcl-2-like protein 4 and tumor protein p53. Phloretin treatment also affected the expression of apoptotic protease activating factor-1, the protein product of the direct binding of the inhibitor of apoptosis protein with low PI to the X-linked inhibitor of apoptosis protein. The present results indicated that phloretin may inhibit EC-109 cell growth by inducing apoptosis, which may be mediated through a mitochondria-dependent pathway.

## Introduction

Esophageal cancer is the eighth-most common type of cancer globally, and due to the poor prognosis associated with esophageal cancer, it is the sixth most common cause of cancer-associated mortality (1). An estimate indicates that, in 2011, there were 3,372,175 incidences of cancer and 2,113,048 cancer mortalities (2). Histologically, esophageal cancer can be divided into squamous cell carcinoma (SCC) and adenocarcinoma. At present, potential curative treatment options include surgical resection, chemotherapy and chemoradiation. However, as esophageal cancer exhibits few characteristic clinical manifestations, the majority of patients are diagnosed at the advanced stage of the disease (3). In the advanced stages of the disease, the tumor will have already metastasized, and the most important types of treatment are chemotherapy or radiotherapy. Currently, chemotherapy usually comprises of cisplatin and 5-fluorouracil-based therapy, but the increase in survival rate is limited and the 5-year survival rate of esophageal cancer remains low (4). Due to high prevalence rate of esophageal cancer, novel approaches are required to prevent and treat the disease. As a result, attention is being paid at present to numerous phytochemicals that are being explored as potential chemopreventive agents that may reverse or suppress esophageal cancer progression.

Apoptosis is the active process of programmed cell death. Apoptosis depends on an intrinsic apoptotic pathway, which occurs in the mitochondria, or on an extrinsic apoptotic pathway, which involves Fas death receptors (5). The B-cell lymphoma (Bcl) protein family regulates the mitochondrial apoptosis pathway by controlling the permeability of the outer mitochondrial membrane. When the expression of the pro-apoptosis protein Bcl-2-associated X protein (Bax) is increased, the pro-survival protein Bcl-2 cannot bind to every Bax protein, and thus apoptosis is triggered (6). Apoptosis stimuli increase the level of expression of p53, due to an increase in the permeability of the outer mitochondrial membrane and the release of the apoptotic protease activating factor (APAF-1) and the protein of the direct inhibitor of apoptosis (IAP) binding protein with low PI (Smac/DIABLO) from the mitochondria (7). Subsequent to the binding of cytochrome c and dATP, APAF-1 forms an oligomeric apoptosome. This apoptosome may stimulate the subsequent caspase

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cascade that commits the cell to apoptosis (8). In addition to this, the Smac/DIABLO protein complex inhibits the binding of X-linked inhibitor of apoptosis protein (XIAP) in order to promote apoptosis (9).

2,4,6-trihydroxy-3-(4-hydroxyphenyl)-propiophenone (phloretin), which can be found in apple tree leaves and the Manchurian apricot, is known to exhibit antioxidative, antimicrobial, anti-inflammatory and antitumor properties (10). Phloretin has been shown to exert antitumor activity through the inhibition of protein kinase C (PKC) activity and the induction of apoptosis (11). The proliferation of the colon cancer HT-29 cells, Fischer bladder cell carcinoma cell lines and Lymphatic tumors have been found to be inhibited by phloretin (12). The effects and anticancer mechanisms of phloretin on esophageal cancer remains unclear. The present study therefore investigated the potential molecular mechanism of phloretin-induced tumor cell apoptosis in EC-109 cells.

## Materials and methods

**Cell culture.** The esophageal cancer EC-109 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 mg/ml penicillin and streptomycin (Invitrogen, Thermo Fisher Scientific, Inc.) in 5% CO<sub>2</sub> at 37.5°C.

**Reagents.** Phloretin, purity >99%, was purchased from Xi'an Plants of Grass Technology Co. Ltd. (Xi'an, China). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Benzyloxycarbonyl (Cbz)-l-Val-Ala-Asp (OMe)-fluoromethylketone (Z-VAD-FMK), electrochemiluminescence (ECL), protease inhibitors (phenylmethanesulfonyl fluoride) and phosphatase inhibitors (PhosSTOP) were purchased from Beyotime Institute of Biotechnology (Beijing, China). Antibodies against BAX (#50599-2-Ig; dilution, 1:500), Bcl-2 (#12789-1-AP; dilution, 1:600), APAF-1 (#21710-1-AP; dilution, 1:200), DIABLO (#10434-1-AP; dilution, 1:1,000), XIAP (#10037-1-Ig; dilution, 1:400) and p53 (#10442-1-AP; dilution, 1:700) were purchased from ProteinTech Group, Inc. (Wuhan, China).

**Cellular proliferation assay.** Cell viability was measured using an MTT assay. A total of 1x10<sup>4</sup> cells were cultured in a 96-well plate and treated with 60-100 µg/ml phloretin for 6, 12, 24 or 48 h. In total, 1 mg/ml of MTT solution (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was then added to the medium, and the cells were incubated for an additional 4 h at 37.5°C. The medium was then removed, and 100 µl dimethyl sulfoxide was added to dissolve the solid residue. The absorbance of each well was read at 540 nm using a micro-ELISA reader. Percent cell survival was defined as the relative absorbance of treated vs. untreated cells.

**Cellular apoptosis assay.** An Annexin V:PE apoptosis detection kit (Beyotime Institute of Biotechnology) was used to measure the number of apoptotic cells subsequent to the

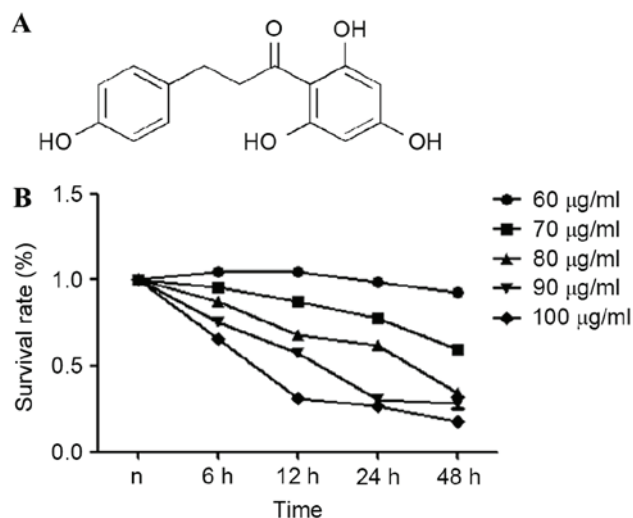


Figure 1. The effect of phloretin, a fruit tree extract, on cell viability. (A) Chemical structure of phloretin. (B) Survival rate of EC-109 cells when treated with phloretin at concentrations ranging from 60-100 µg/ml for 6, 12, 24 and 48 h. The cell viability was quantified using an MTT assay.

cells being treated with phloretin at different concentrations of 60, 70 and 80 µg/ml for 12 h. The cells were trypsinized and washed twice with cold PBS, and re-suspended in 1% bovine serum albumin solution with 5 µl Annexin V:PE and 5 µl 1-7-AAD at a concentration of 1x10<sup>5</sup> ml/cells in a total volume of 100 µl. The cells were gently mixed, and incubated in the dark for 15 min at room temperature. A quota of 1 µl for Annexin V:PE apoptosis detection kit (#C1062; Beyotime Institute of Biotechnology) was subsequently added to each test tube and the number of apoptotic cells was quantified by flow cytometry using the BD LSR II Analyzer (BD Biosciences, Franklin Lakes, NJ, USA) within 1 h.

**Western blot analysis.** The expression levels of the cellular proteins were determined using western blotting assays. The EC-109 cells were treated with 60 µg/ml phloretin for 6, 12 and 24 h. The EC-109 cells were washed with PBS at 4°C subsequent to treatment with phloretin, and lysed with 200 µl radioimmunoprecipitation assay buffer (50 mmol 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid at pH 7.5, 150 mmol NaCl, 10% glycerol, 1.5 mmol MgCl<sub>2</sub>, 1% Triton-X 100, 1 mmol EDTA at pH 8.0, 10 mmol sodium pyrophosphate and 10 mmol sodium fluoride) containing a mixture of protease inhibitors (phenylmethanesulfonyl fluoride; 1:100) and a mixture of phosphatase inhibitors (PhosSTOP; 1:40; all, Beyotime Institute of Biotechnology). The present study used the Pierce BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) to measure protein concentrations. Equal amounts of protein samples were electrophoresed on 8-12% SDS-PAGE mini-gel subsequent to thermal denaturation for 10 min at 100°C. The proteins were then transferred onto a polyvinylidene fluoride membrane at 200 mA for 2 h at 4°C. The membranes were probed with the previously indicated antibodies with the previously indicated concentrations overnight at 4°C, then blotted with a horseradish peroxidase-conjugated cow anti-rabbit secondary antibody (ProteinTech Group, Inc.). Visualization of the membranes was performed using the Bio-Rad ChemiDoc XRS system (Bio-Rad Laboratories,

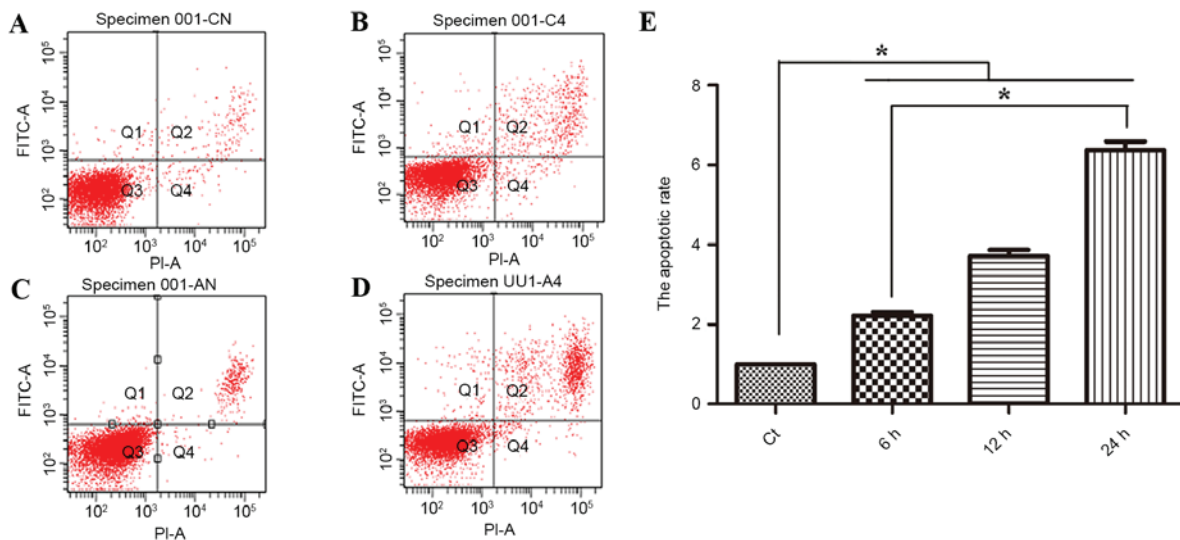


Figure 2. Flow cytometry analysis and histograms. Cell cycle distribution profiles are included for (A) the Ct group and cells treated with phloretin at (B) 60, (C) 70 and (D) 80  $\mu\text{g/ml}$  for 12 h. (E) A bar graph demonstrating the rate of apoptosis using flow cytometry analysis. Data represent the mean  $\pm$  standard deviation of 3 independent experiments. \* $P < 0.05$  between indicated groups, unpaired Student's t-test. FITC-A, fluorescein isothiocyanate-A; PI-A, propidium iodide-A, Ct, control.

Inc., Hercules, CA, USA) with ECL (Beyotime Institute of Biotechnology). The western blot was repeated three times, and the blots were quantitatively analyzed using Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** All the presented data were confirmed in a minimum of 3 independent experiments, and are expressed as the mean  $\pm$  standard deviation. Statistical comparisons were made by unpaired Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Phloretin causes dose-dependent and time-dependent growth inhibition in EC-109 cells.** Chemical structure of phloretin is presented in Fig. 1A. An MTT assay was used to estimate the effects of phloretin on the viability of the EC-109 cells. From the results obtained, it was demonstrated that the survival rate of the EC-109 cells treated with 60–100  $\mu\text{g/ml}$  phloretin for 6, 12, 24 and 48 h significantly reduced in a dose- and time-dependent manner, as illustrated in Fig. 1B.

**Phloretin induces apoptosis in EC-109 cells.** Subsequent to the confirmation that the treatment of human esophageal cancer with phloretin resulted in a reduction in cell viability, the present study then investigated whether the effect of phloretin is associated with apoptosis. As revealed in Fig. 2A, flow cytometric analysis suggested that subsequent to treatment with 60, 70 and 80  $\mu\text{g/ml}$  phloretin for 12 h, the apoptotic index of the EC-109 cells increased to  $225.6 \pm 16.0$ ,  $375 \pm 24.7$  and  $634 \pm 44.6\%$ , respectively (\* $P < 0.05$ , compared with the standard control group).

**Phloretin induces changes in the expression levels of the Bcl protein family.** Following on from the observation of the regulatory effect of phloretin on the apoptosis in the EC-109 cells, the present study then examined the effect of phloretin

on the expression of the key apoptotic regulators Bax and Bcl-2. Fig. 3A provides statistical calculations of the Bcl protein family expression ratios, demonstrating an increase in the level of Bax, whilst Fig. 3B demonstrates a decline in Bcl-2 levels that consequently led to the increase of the relative rate of Bax/Bcl-2 which evidently occurred in a time-dependent manner.

**Phloretin causes an increase in the expression levels of p53.** The induction of apoptosis is a key aspect of the tumor-suppressive activity of p53. Fig. 4A demonstrates that treatment with 60  $\mu\text{g/ml}$  phloretin for 6, 12, and 24 h induced a time-dependent increase in p53 activity, with increases of  $178.1 \pm 17.6$ ,  $249.5 \pm 18.7$  and  $261.6 \pm 17.6\%$  respectively in EC-109 cells (\* $P < 0.05$ , compared with the standard control group).

**Mitochondrial apoptotic pathways are involved in the anticancer mechanism of phloretin.** To investigate the potential underlying mechanism of the proapoptotic activities of phloretin on EC-109 cells, the present study detected changes in the proteins within the mitochondrial apoptotic pathways. Smac/DIABLO and APAF-1, which were located the mitochondrial intermembrane space, were pro-apoptotic with respect to phloretin. Fig. 5A demonstrates that treatment with 60  $\mu\text{g/ml}$  phloretin for 6, 12 and 24 h induced a time-dependent increase in DIABLO activity, with increases of  $225.1 \pm 5.6$ ,  $280.5 \pm 15.7$  and  $351.6 \pm 17.6\%$ , respectively in EC-109 cells, ( $P < 0.05$ , compared with the standard control group). Fig. 5B demonstrates that levels of APAF-1 also increased significantly to  $160 \pm 9.9$ ,  $245.5 \pm 15.2$  and  $260.4 \pm 10.4\%$ , respectively in the EC-109 cells, subsequent to the different melatonin treatments ( $P < 0.05$ , compared with the standard control group). XIAP is a member of the IAP family of proteins and inhibits apoptotic cell death. Fig. 5C demonstrates that subsequent to treatment with 60  $\mu\text{g/ml}$  phloretin for 6, 12 and 24 h, a time-dependent decrease in the levels of XIAP was observed in the EC-109

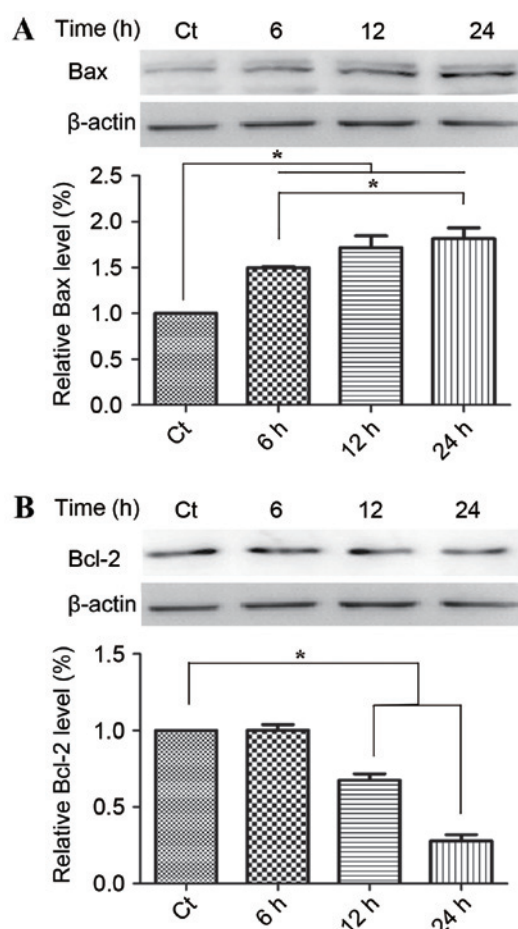


Figure 3. The relative expression level of cells treated with phloretin, as determined by western blot and relative quantification of (A) Bax and (B) Bcl-2. Data represent the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  between indicated groups, unpaired Student's t-test. Ct, control; Bax, Bcl-2-associated X protein.

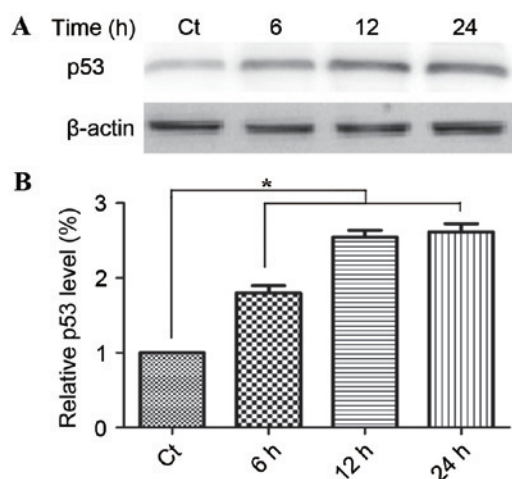


Figure 4. The relative p53 expression level of cells treated with phloretin. (A) Western blotting was used to detect p53. (B) The western blot image was then used for relative quantification. \* $P < 0.05$  between indicated groups, unpaired Student's t-test. Ct, control.

cells, with intracellular levels of XIAP measuring  $84.3 \pm 4.4$ ,  $75 \pm 7.0$  and  $57 \pm 6.4\%$ , respectively ( $P < 0.05$ , compared with the standard control group).

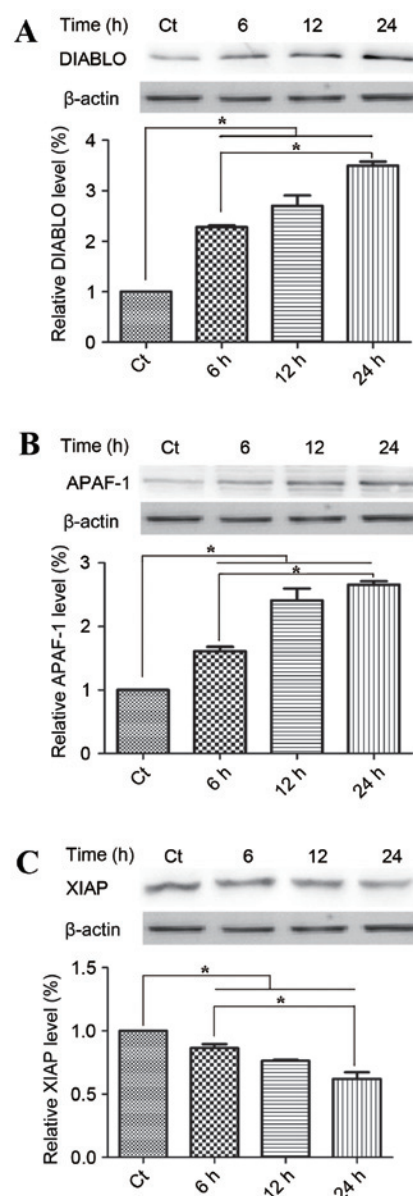


Figure 5. The relative expression level of cells treated with phloretin, as determined by western blot and relative quantification of (A) Smac/DIABLO, (B) APAF-1 and (C) XIAP. \* $P < 0.05$  between indicated groups, unpaired Student's t-test. Ct, control; DIABLO (Smac/DIABLO), direct binding protein with low PI; APAF-1, apoptotic protease activating factor 1; XIAP, X-linked inhibitor of apoptosis protein.

## Discussion

In 1993, Nelson and Falk (13) reported that phloretin restricts tumor cell growth by inhibiting glucose transmembrane transport. Furthermore, there is support for the hypothesis that phloretin serves a pivotal role in numerous anticancer mechanisms. Using an MTT assay and flow cytometric analysis, the present study demonstrated that phloretin exhibits antitumor behaviors, including the inhibition of proliferation and the induction of apoptosis. Apoptosis, programmed cell death, is required to maintain homeostasis. Due to decreased rates of apoptosis, tumor cells can survive indefinitely. Several studies have revealed that anticancer drugs induce apoptosis, and thus inhibit the proliferation of the tumor cells (14,15). At present, there are numerous drugs, including drugs targeting the



caspase family of proteins, which can regulate the apoptotic pathway (16).

The Bcl protein family serves an important role in apoptosis. This family, which is termed due to their different functions and structural homology, may be divided into proapoptotic and antiapoptotic proteins. The antiapoptotic proteins include Bcl-2, Bcl-extra large (Bcl-xL), Bcl-2-like protein 2 (Bcl-w) and myeloid leukemia cell differentiation protein (Mcl-1). Equally, proapoptotic proteins include Bax and Bcl-2 homologous antagonist killer (Bak), which can provoke mitochondrial damage and promote cell apoptosis (17). In the present study, western blot analysis revealed that when phloretin stimulated the EC-109 cells, the expression of Bax protein increased, which was accompanied by a decline in the expression of Bcl-2. Under normal circumstances, levels of the pro-apoptosis protein Bax and the anti-apoptosis protein Bcl-2 will maintain the balance of apoptosis. The survival rates of tumor cells depend on the level of Bcl-2 protein present. The expression of Bcl-2 in tumor cells is increased in comparison with the expression of Bcl-2 expression within normal cells. Bcl-2 protects cells from apoptosis, in part due to their ability to bind the Bcl-2 homology (BH) 3-exposed conformers of Bax and Bak, thereby inhibiting full activation of the aforementioned proteins. When the level of Bcl-2 decreases and Bcl-2 cannot bind to Bax, cells will trigger apoptosis. The proapoptotic proteins Bax and Bak serve an important role in the induction of caspase activation (18). Activated Bax inserts into the mitochondrial membrane and increases membrane permeability, leading to an activation of the mitochondrial apoptotic pathway.

p53, as a transcription factor, serves as a tumor suppresser protein and initiates cell death via the mitochondrial apoptotic pathway (19). A previous study revealed that p53 interacts with various members of the Bcl protein family (20). In the cytosol, p53 forms an inhibitory complex with Bcl-2 to induce cell death, whilst the proapoptotic protein Bax may be directly activated to induce cell death. Activated Bax may form homooligomers, which participate in forming pores and in the control of the permeabilization of the outer mitochondrial membrane, leading to the release of the mitochondrial intermembrane space into the cytosol, including cytochrome *c*, APAF-1 and Smac/DIABLO proteins (21). It has been revealed that when EC-109 cells are treated with phloretin, levels of Smac/DIABLO and APAF-1 increase.

APAF-1, which is assembled into a ring-like platform, is the central component of the apoptosome. In the presence of dATP/ATP, the cytochrome *c* released from mitochondria interacts with the APAF-1 proteins, initiating the formation of the apoptosome. The apoptosome subsequently begins to recruit pro-caspase-9, and activates the pro-caspases in the intrinsic cell-death pathway, in order to initiate apoptosis via nucleus condensation and/or the degradation of other intracellular structures (22).

Smac/DIABLO is released from the mitochondria, and along with cytochrome *c*, is another important proapoptotic factor. In contrast, XIAP is a member of the IAP family of proteins, and inhibits apoptotic cell death. Smac/DIABLO neutralizes the inhibitory activity of XIAP to promote cytochrome *c*-APAF-1-dependent caspase activation (9). From western blot analyses there is a growing body of evidence

indicating that phloretin increases the levels of the proteins p53, APAF-1 and DIABLO, and reduce the levels of XIAP protein to induce apoptosis. Previous studies combined with the results of the present study, have revealed that phloretin increases the levels of apoptosis of EC-109 cells via the mitochondria-apoptotic pathway (23-25).

In conclusion, the present study has reported that phloretin inhibit the proliferation of human esophageal cancer EC-109 cells, and indicated that phloretin exhibits anticancer behavior through the activation of the mitochondrial-apoptosis pathway. In summary, phloretin may be considered to be a promising chemopreventive agent for the treatment of esophageal cancer.

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