

Full Paper

Phaseoloideside E, a Novel Natural Triterpenoid Saponin Identified From *Entada phaseoloides*, Induces Apoptosis in Ec-109 Esophageal Cancer Cells Through Reactive Oxygen Species GenerationShasha Mo^{1,†}, Hui Xiong^{1,†}, Guangwen Shu¹, Xinzhou Yang¹, Jianxia Wang¹, Congyi Zheng², Wei Xiong², and Zhinan Mei^{1,*}¹College of Pharmacy, South Central University for Nationalities, Wuhan 430074, P.R. China²State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, P.R. China

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Abstract. Phaseoloideside E (PE), a new oleanane-type triterpene saponin, was isolated from the seed kernels of *Entada phaseoloides* (Linn.) MERR. PE had strong cytotoxic activity against an array of malignant cells. Typical morphological and biochemical features of apoptosis were observed in PE-treated Ec-109 cells. PE induced a dose-dependent increase in the sub-G1 fraction of the cell cycle and DNA fragmentation. Decreases in the mitochondrial membrane potential, SOD activity, and GSH content were also observed. Further investigations revealed that PE reduced the ratio of Bcl-2 to Bax and increased the activities of caspase-3 and -9, but this was prevented by Z-VAD-fmk. PE also induced a decrease of the sub-G1 fraction. Furthermore, PE-induced apoptosis was mediated by up-regulating cellular ROS, which was suppressed by cotreating the cells with *N*-acetylcysteine (NAC). NAC also attenuated the ratio of sub-G1, the generation of DNA fragmentation and the expression of Bcl-2, Bax, caspase-3, and caspase-9. Interestingly, PE did not up-regulate ROS or induce cell death in untransformed cells. These data showed that PE induces cell death through up-regulation of cellular ROS production. Our investigation provides the scientific basis for the traditional application of this herb and suggests the possibility that PE may be used for a treatment of esophageal carcinoma.

[Supplementary materials: available only at <http://dx.doi.org/10.1254/jphs.12193FP>]

Keywords: *Entada phaseoloides*, natural compound, esophageal cancer, apoptosis, reactive oxygen species

Introduction

Entada phaseoloides (Linn.) MERR., a member of the genus *Entada* (Family Leguminosae), is commonly found in southern China. It was documented in earlier Chinese medical material, such as “Nanfang Caomu Zhuang” (Jin dynasty, about 1700 years ago) and “Bencao Gangmu” (Ming dynasty, about 600 years ago), that it has been used as a folk medicine for the treatment of cancer, diabetes mellitus, hyperlipidemia, and stomach ache. We have reported that the total saponins from

E. phaseoloides (L.) MERR. had an anti-diabetic effect in type 2 diabetic rats (1). In addition, the anti-tumor activity of saponin extract from *E. phaseoloides* was investigated (2). However, the chemical constituents underlying the anticancer activity of this plant are poorly understood. Investigations revealed the presence of a series of natural products such as phenylacetic acid derivatives (3), sulfur-containing amides (4–5), and oleanane-type triterpene saponins (6–9). Saponin is an abundant type of secondary metabolic product found in the seed of this plant. Some biological activities of saponins from diverse origins have been reported including cytotoxic effects toward different types of cancer cells (7–10). These data imply the possibility that some saponins are responsible for the anticancer activity of this herb.

[†]The first two authors contributed equally to this work.*Corresponding author. meizhinan@163.com

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Esophageal cancer (EC) is a very common malignancy in the digestive tract found worldwide, and it is particularly prevalent in some parts of China. It is one of the most aggressive types of human cancer and a major cause of cancer-related death. Chemotherapy is a standard EC treatment, with fluoropyrimidines, taxanes, and platinum compounds being the most frequently used chemotherapeutic reagents (11). Unfortunately, the general death rate of EC patients still remains high (60%–90%) owing to metastasis, advanced disease, and tumor drug tolerance (12). Therefore, there is a great need for identifying novel therapeutic drugs against EC.

Recently, more attention has been paid to the natural products from traditional herbs as a potential source of new therapeutic drugs (13–15). Our previous studies revealed that the *n*-BuOH soluble fraction of the 70% ethanolic extract of the seed kernels of *E. phaseoloides* had the strongest in vitro anticancer activity compared with other fractions (petroleum ether, ethyl acetate, and water fractions) (unpublished data). This observation suggested that some compounds in the *n*-BuOH fraction were implicated in the anticancer activity of this folk medicine.

In this study, we isolated and identified a novel oleanane-type triterpene saponin named phaseoloideside E (PE) from the *n*-BuOH fraction of the seed kernels of *E. phaseoloides*. PE was effective at inducing apoptotic cell death against human Ec-109 esophageal cancer cells. Interestingly, untransformed human L-O2 hepatocytes and murine 3T3-L1 fibroblasts were resistant to PE treatment at the same concentration. Moreover, we demonstrated that PE induced Ec-109 cell apoptosis in a caspase-dependent manner. Further investigations revealed that PE induces cell death through up-regulation of cellular ROS production. These data provided a scientific basis for the traditional application of this herb and suggested the possibility that PE has potential therapeutic value against esophageal carcinoma.

Materials and Methods

Plant material, drugs, and chemicals

The seed kernels of *E. phaseoloides* were collected in Xishuangbanna, Yunnan Province, China and botanically verified by Prof. Dingrong Wan, College of Pharmacy, South-Central University for Nationalities. The location is not privately-owned or protected in any way. No specific permissions were required for the field studies described above, and they did not involve endangered or protected species. A voucher specimen (No. EP-200802) was deposited in the herbarium of the College of Pharmacy, South-Central University for Nationalities.

MTT and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA); Z-VAD-fmk, *N*-acetylcysteine (NAC), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), and JC-1 were purchased from Beyotime Biotechnology (Nantong, China); all the primary and secondary antibodies were from Cell Signaling Technology (Danvers, MA, USA). The HPLC grade chemical reagents were purchased from Tedia (Fairfield, OH, USA). All other chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Isolation and characterization of PE

Dried powdered seed kernels (10 kg) were extracted four times at room temperature using 70% ethanol. After extracting with petroleum benzene and *n*-BuOH, the *n*-BuOH extract was subjected to column chromatography over HP 20 and eluted with H₂O/EtOH gradients. The total saponins of *E. phaseoloides* (TSEP, 61.6 g) were obtained from the 50% H₂O/EtOH fraction. TSEP was further subjected to silica gel column chromatography and eluted with EtOAc-MeOH-H₂O gradients (100:0:0, 80:20:0, 60:40:8, 40:60:10, 0:100:0) to give Fr.1 (collected at a 60:40:8 gradient). Fr.1 was further subjected to column chromatography (ODS, 50 μ m, H₂O/MeOH) to give the fractions containing PE. The purity of each fraction was assessed by semi-preparative HPLC (DIONEX Ultimate 3000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with isocratic H₂O/MeCN gradients (72/28, v/v, 3.0 ml/min, 212 nm). The structure of the compound was elucidated by spectral analyses (¹H-NMR, ¹³C NMR, and HR-ESI-MS).

Cell lines and cell culture

Ec-109, L-O2, and 3T3-L1 cells were purchased from the China Type Culture Collection Center (CCTCC; Wuhan, China). All the cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin, and streptomycin and cultured at 37°C in a humidified atmosphere supplied with 5% CO₂.

Cell viability assay and IC₅₀ value calculation

Cells were seeded on 96-well plates and allowed to adhere overnight. After the indicated treatments, the MTT assay was performed by replacing the medium with 10 μ L of medium containing MTT (5 mg/mL) followed by incubating the cells at the standard culture condition for 3 h. Then, 150 μ L of DMSO was added to each well and mixed thoroughly. The optical density (OD) was read at 570 nm.

The IC₅₀ value of a compound is defined as the concentration at which it can repress cell viability by 50%.

It was calculated from a plot of the cell viability versus the compound concentration using Origin Software.

Assessing the PE that enters the cell

After incubating Ec-109 cells with PE (60 $\mu\text{g/mL}$) for 24 h, the medium was discarded and the cells were washed three times with ice-cold $1 \times \text{PBS}$. Then the cells were harvested and resuspended in $1 \times \text{PBS}$. The cell suspensions were centrifuged at $1500 \times g$ for 10 min. The supernatants were subjected to HPLC. No PE signal could be detected in the supernatants if the cells were intact, indicating that the PE outside the cells was removed by washing. Then, the cell pellet was homogenized by ultrasonication in $1 \times \text{PBS}$ and centrifuged again at $12000 \times g$ for 15 min. The supernatants were analyzed by HPLC.

Acridine orange/ethidium bromide (AO/EB) staining

Ec-109 cells (1×10^5 cells per well) were seeded in a 12-well plate and treated with PE at different concentrations for the indicated time. Cells were stained by AO (2 $\mu\text{g/mL}$) and EB (2 $\mu\text{g/mL}$). The morphological changes of the cells were observed under an inverted phase-contrast microscope. The photographs were taken at $400 \times$ magnification. Live cells were stained green by AO, and the apoptotic cells were stained orange or red by EB.

Flow cytometry analysis (FACS)

After the indicated treatment, Ec-109 cells were trypsinized and incubated with 70% ethanol overnight at 4°C for fixation. Cells were then pelleted and washed with PBS containing 20 mM EDTA. RNA was removed by incubating samples with RNase A (1 mg/mL) at 37°C for at least 1 h. Cells were then stained with propidium iodide (PI) at a final concentration of 30 $\mu\text{g/mL}$. PI-positive cells were detected by FACS (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA). The amount of apoptotic cells was determined by the percentage of sub-G1 DNA content in each sample.

DNA fragmentation assay

Cells were rinsed with PBS twice and lysed on ice for 30 min in 10 mM Tris-HCl (pH 8.0), 25 mM EDTA, and 0.25% Triton X-100. After centrifugation at $12,000 \times g$ for 15 min, the supernatant was incubated with RNase A at 37°C for 1 h and then with Proteinase K at 56°C for 30 min. The DNA content was extracted sequentially with phenol, phenol: chloroform (1:1), and chloroform. The DNA in the aqueous phase was precipitated, separated by 1.5% agarose gel electrophoresis, and then visualized and photographed under transmitted UV light.

Western blotting assay

Cells cultured in 35-mm dishes were lysed in 0.2 mL of ice-cold lysis buffer containing 50 mM Hepes (pH 7.4), 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4 , 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 1 mM PMSF. The lysates were centrifuged at $12,000 \times g$ for 15 min. The supernatants were collected and protein concentrations were determined by Bradford's method. Proteins (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. The membranes were blocked for 30 min with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and subsequently incubated with primary antibody (1:2000 dilution) overnight at 4°C . After washing with TBST for 30 min at room temperature, the membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h, followed by 45 min of washing (with three to five changes of the wash buffer). Protein bands were finally visualized by enhanced chemiluminescence (ECL).

Detection of intracellular reactive oxygen species (ROS) generation

The cells were seeded at a density of 2×10^5 in a 6-well plate and then incubated for 24 h. After the indicated treatments, the cells were incubated with the ROS fluorescent probe DCFH-DA at a final concentration of 10 μM at 37°C for 30 min and then washed three times with PBS. Cellular ROS levels were determined by fluorescence microscope observation with excitation at 488 nm.

Detection of glutathione (GSH), total superoxide dismutase (T-SOD), and Cu-Zn SOD levels in Ec-109 cells

Cells were collected as mentioned above, and then lysed in 0.2 mL of ice-cold lysis buffer. The GSH, T-SOD, and Cu-Zn SOD levels were determined using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

Detection of mitochondrial membrane potential ($\Delta\psi\text{m}$)

Cells treated with PE for the indicated concentration and time were incubated with JC-1 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 30 min, washed with PBS, and then observed using a fluorescence microscope. The photographs were taken at $400 \times$ magnification.

$\Delta\psi\text{m}$ was also determined by flow cytometry. Cells were treated with PE for the indicated concentration and time. Cells were collected, washed, and incubated with JC-1 for 30 min. Cells were centrifuged, washed

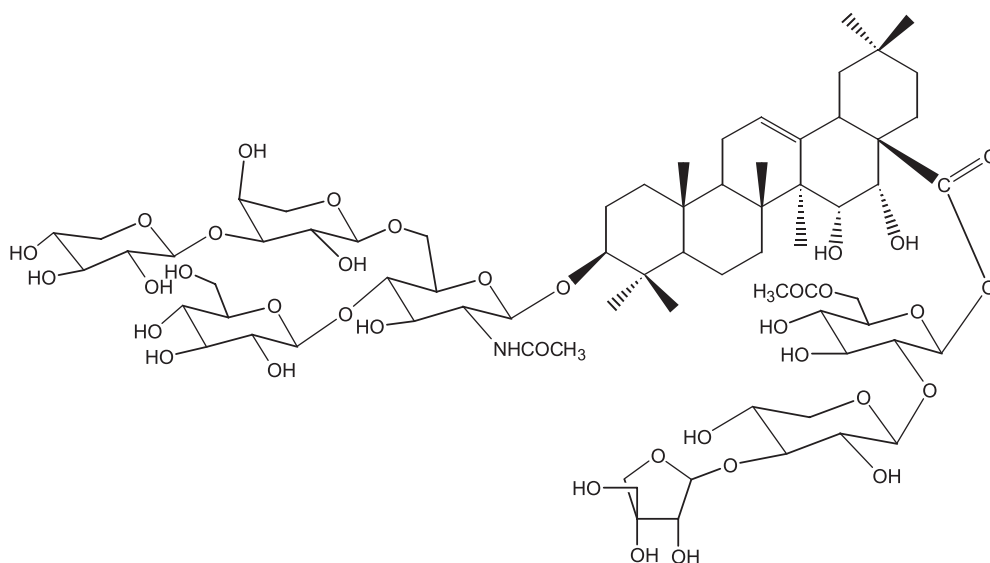


Fig. 1. Chemical structure of Phaseoloideside E.

and suspended in PBS, and then analyzed by flow cytometry.

All results shown in this paper are representatives of the mean value $\pm$ S.E.M. of at least three independent experiments.

Results

Identification of a new compound from *E. phaseoloides*

An active *n*-BuOH fraction prepared from the 70% EtOH extract of the seed kernels of *E. phaseoloides* was subjected to repeated column chromatography on HP20 and ODS, followed by semi-preparative HPLC to yield a new compound PE. The chemical structure of the new compound is shown in Fig. 1.

PE was obtained as a white amorphous powder. The molecular formula of PE was determined to be $C_{72}H_{115}NO_{37}$ by the positive HRESIMS $[M + Na]^+$ ion at m/z 1608.7019 (calcd 1608.7040) and NMR data. Its negative FABMS showed the pseudomolecular ion peak at m/z 1584 $[M-1]^-$. The ion peak of further fragments were observed on the FAB-MS at m/z 1452 $[M-132-1]^-$, 1422 $[M-162-1]^-$, 1320 $[M-162-132-1]^-$, and 1116 $[M-162-132-132-42-1]^-$. The 1H and ^{13}C NMR data of PE (Supplementary Table 1: available at online version only) and rheediinose B (10) were almost identical, suggesting that these two compounds had the same aglycon and sugar chains at C-3 and C-28. The only difference was the presence of an additional acetyl group at C-6 of Glc II instead of a proton as found in rheediinose B, which was further confirmed by the ROESY and HMBC data. Therefore, PE was elucidated to be 3-*O*- β -D-glucopyranosyl-(1-4)-

$[\beta$ -D-xylopyranosyl-(1-3)- α -L-arabinopyranosyl-(1-6)]-2-acetylamino-2-deoxy- β -D-glucopyranosylentagenic acid 28-*O*- β -D-apiofuranosyl-(1-3)-*O*- β -D-xylopyranosyl-(1-2)-(6-*O*-acetyl)- β -D-glucopyranoside. The purity of PE was more than 95% as determined by HPLC.

Cytotoxic effects of these compounds toward malignant and untransformed cells

Based on the fact that the *n*-BuOH fraction of *E. phaseoloides* showed cytotoxic activities toward various human cancer cell lines, the in vitro anticancer activity of PE was tested against four human cancer cell lines including HepG-2 hepatocellular carcinoma cells, Ec-109 esophageal cancer cells, SW480 colon carcinoma-derived cells, and HeLa cervical cancer cells. As shown in Table 1, PE was a potent cancer cell-death inducer. Among all the cancer cell lines tested, Ec-109 cells were the most sensitive to PE administration. In contrast, two untransformed cells, human L-O2 hepatocytes and murine 3T3-L1 fibroblasts, were resistant to it.

PE permeated Ec-109 cells

To explore whether PE could act intracellularly, we performed an HPLC assay (Supplementary Fig. 1: available at online version only). We treated Ec-109 cells with vehicle and PE (60 μ g/mL). After 24 h, total cell lysates were prepared and subjected to HPLC using purified PE as a control. PE was detected by HPLC both in the control sample and intracellular sample, but was not found in the intracellular blank sample. This result indicated that PE was membrane permeable and could penetrate into the cells.

Table 1. Cytotoxicity of Phaseoloideside E

Compounds	IC ₅₀ (μ M)					
	HepG-2	SW480	Ec-109	HeLa	L-O2	3T3-L1
PE	55.0 $\pm$ 4.1	23.3 $\pm$ 2.4	25.3 $\pm$ 2.6	41.6 $\pm$ 3.2	> 100	> 100
Cisplatin	25.5 $\pm$ 3.4	19.7 $\pm$ 2.9	25.5 $\pm$ 2.1	20.8 $\pm$ 2.5	35.1 $\pm$ 5.7	39.7 $\pm$ 4.2

The data are given as the mean $\pm$ S.E.M., n = 3. > 100 = inactive.

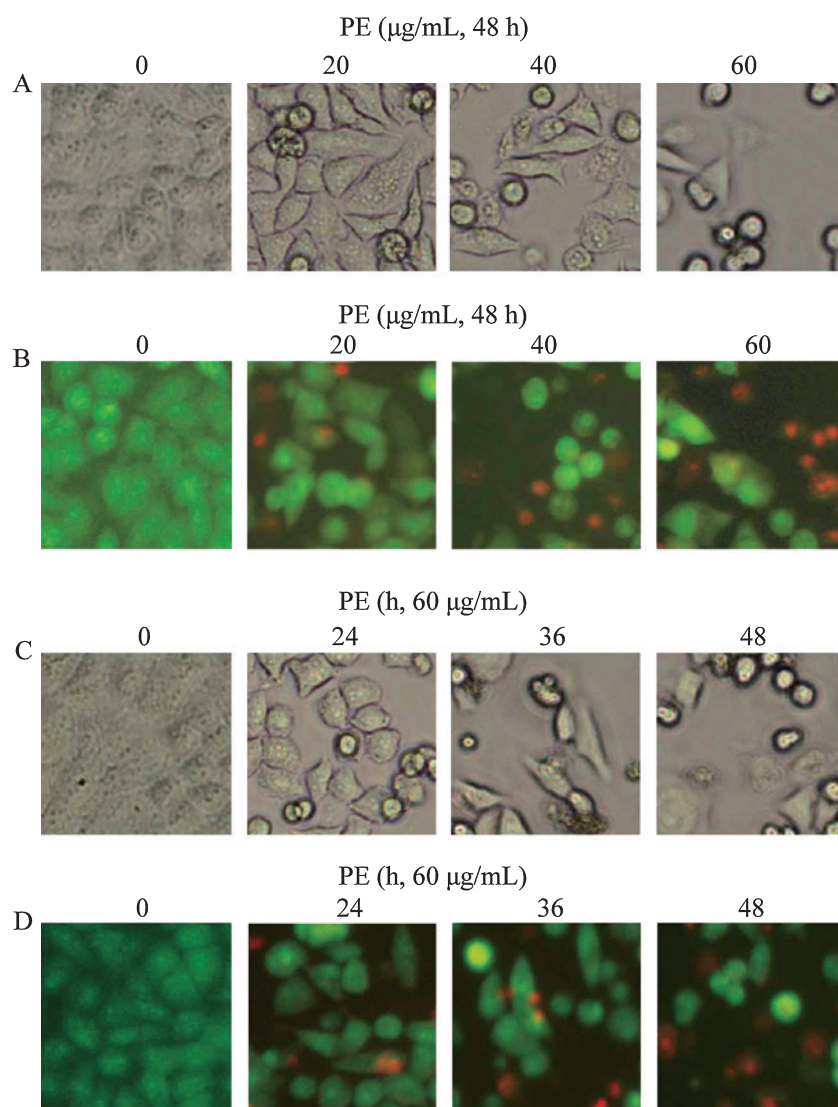


Fig. 2. Morphologic examinations during PE-induced Ec-109 cell death. Ec-109 cells were treated with different concentrations of PE for 48 h by contrast observation (A) and AO/EB staining (B). Cells were treated with 60 μ g/mL of PE for the indicated times by contrast observation (C) and AO/EB staining (D).

PE-induced morphologic changes in Ec-109 cells

To address the pattern of PE-induced Ec-109 cell death, the morphology of Ec-109 cells before and after PE treatment was directly examined using an inverted microscope. As shown in Fig. 2, A and C, as the drug concentration and treatment time increased, more cells shrunk and detached from the culture plate. We also performed an AO/EB fluorescence staining assay. The

result showed that the ratio of EB-positive cells increased dose- and time-dependently (Fig. 2: B and D). These data suggested that PE induced apoptosis-related morphological alterations in Ec-109 cells.

PE triggers caspase-dependent apoptosis in Ec-109 cells

To confirm the role of the apoptosis pathway in PE-mediated Ec-109 cell death, we performed FACS analy-

sis. The results showed that PE treatment dose- and time-dependently increased the ratio of apoptotic cells (Fig. 3: A and B). Moreover, DNA fragmentation, another important characteristic of cell apoptosis, was also observed (Fig. 3C). Bcl-2 family proteins, such as anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, play important roles in modulating cell apoptosis (16–17). A western blotting assay revealed that PE dose-dependently decreased Bcl-2 and increased Bax protein levels in Ec-109 cells. Caspases are a family of cysteine proteases. The activation of some members of this family, especially caspase-3, is frequently involved in cell apoptosis (18–19). Consistently, PE-induced up-regulation of activated caspase-3 and caspase-9 were also observed (Fig. 3D). Furthermore, Z-VAD-fmk, a caspase inhibitor (20), effectively repressed PE-mediated Ec-109 cell apoptosis as shown by AO/EB staining (Fig. 3E) and FACS assay (Fig. 3F). Taken together, these results suggested that PE-induced Ec-109 cell death was mediated by a caspase-dependent apoptosis pathway.

PE up-regulates cellular ROS level in Ec-109 cells

A fluorescence staining assay demonstrated that PE treatment dose- and time-dependently increased the cellular ROS level in Ec-109 cells (Fig. 4: A and B), but did not affect ROS levels in L-O2 and 3T3-L1 cells, which were resistant to PE treatment (Fig. 4: C and D).

ROS generation is required for PE-mediated Ec-109 cell apoptosis

To further confirm the role of ROS generation in PE-mediated Ec-109 cell apoptosis, we examined the effect of NAC, an ROS scavenger, on this process. As expected, NAC significantly decreased PE-induced ROS generation (Fig. 5A). Moreover, PE-induced apoptosis was significantly suppressed by NAC as shown by FACS, AO/EB staining, and DNA fragmentation assays (Fig. 5: B–D). Consistently, NAC dramatically attenuated PE-induced caspases-3 and caspase-9 activation and down-regulated the ratio of Bcl-2 to Bax (Fig. 5E). Collectively, these data demonstrated that PE-induced Ec-109 cell apoptosis was mediated by up-regulating the cellular ROS level.

Effects of PE on GSH, T-SOD, and Cu-Zn SOD levels in Ec-109 cells

GSH and SOD are the main components of the cellular anti-oxidant system. To understand the molecular mechanisms underlying the ROS-inducing activity of PE, we determined the effects of PE on the levels of GSH and SOD. In Ec-109 cells, PE treatment time- and dose-dependently decreased the levels of GSH and T-SOD,

although the activity of Cu-Zn SOD was only slightly increased (Fig. 6: A and B). In murine 3T3-L1 fibroblasts, the basal levels of GSH content and T-SOD activity were much lower than those in Ec-109 cells, and PE administration did not change them (Fig. 6C).

Effects of PE on $\Delta\psi_m$ in Ec-109 cells

To test whether ROS accumulation in Ec-109 cells affected mitochondria membrane potential, we measured the membrane potential using the JC-1 staining assay. JC-1 is a mitochondria membrane potential sensitive fluorescent probe. JC-1 enters mitochondria and reversibly changes color from orange to green as the membrane potential decreases. A fluorescence staining assay demonstrated that PE treatment dose- and time-dependently decreased the ratio of orange to green components (Fig. 7: A and B). A flow cytometry assay also showed that the $\Delta\psi_m$ was reduced in a dose- and time-dependent manner (Fig. 7: C and D).

Discussion

E. phaseoloides is commonly used to treat cancers in the digestive tract as a traditional folk medicine by local people in parts of China, Thailand, and Myanmar. To understand the chemical components underlying its anticancer activity, we performed bioassay-guided fractionation and isolation of this herb. Our phytochemistry investigations resulted in the identification of a new oleanane-type triterpene saponin.

Initial cytotoxic evaluations on several cancer cell lines disclosed that the triterpenoid saponin was a significant contributor to the anticancer activity of this herb. In addition, we showed that PE, a large compound with a glucose moiety, could enter into cells using an HPLC assay. This result is consistent with previous reports showing that saponins such as ginsenoside, paeoniflorin, and soyasaponin could be absorbed by Caco-2 cells (21–23). Receptor-mediated endocytosis is a possible mechanism by which a large compound like PE enters cells. There are several carbohydrate moieties in PE that have the potential to associate with receptor proteins located on the plasma membrane and facilitate entry into the cell via endocytosis. Moreover, PE also has the potential to enter cells through a direct interaction with the plasma membrane of mammalian cells (24). PE exhibits a strong in vitro anticancer activity against human Ec-109 esophageal cancer cells. Interestingly, the cytotoxic effect of this individual compound to untransformed cells was very low. This observation suggests that PE-mediated cell death appears to be highly specific to EC cells. Therefore, our current data provide a scientific basis for the anti-tumor effect of this herb and sup-

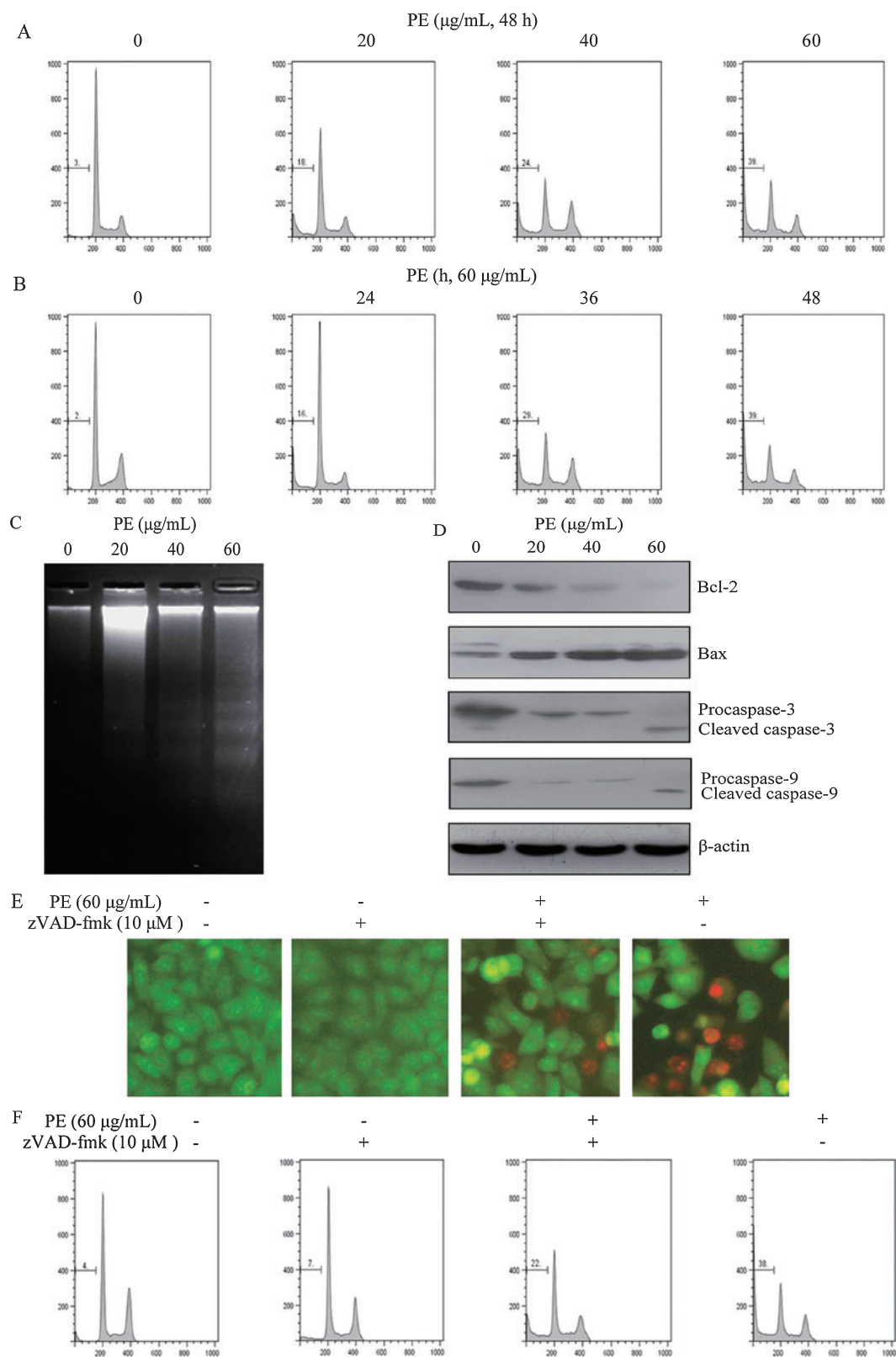


Fig. 3. PE induced Ec-109 cell apoptosis. FACS assay of cells treated with different concentrations of PE for 48 h (A) or 60 $\mu\text{g/mL}$ PE for the indicated times (B). DNA fragmentation assay results of cells treated with the indicated concentrations of PE for 48 h (C) and western blotting analysis of the protein levels of Bcl-2, Bax, caspase-3, and caspase-9 (D). E and F: Ec-109 cells were treated with 60 $\mu\text{g/mL}$ of PE in the presence or absence of zVAD-fmk for 48 h. Then cell apoptosis was determined by AO/EB staining (E) and FACS (F) assay.

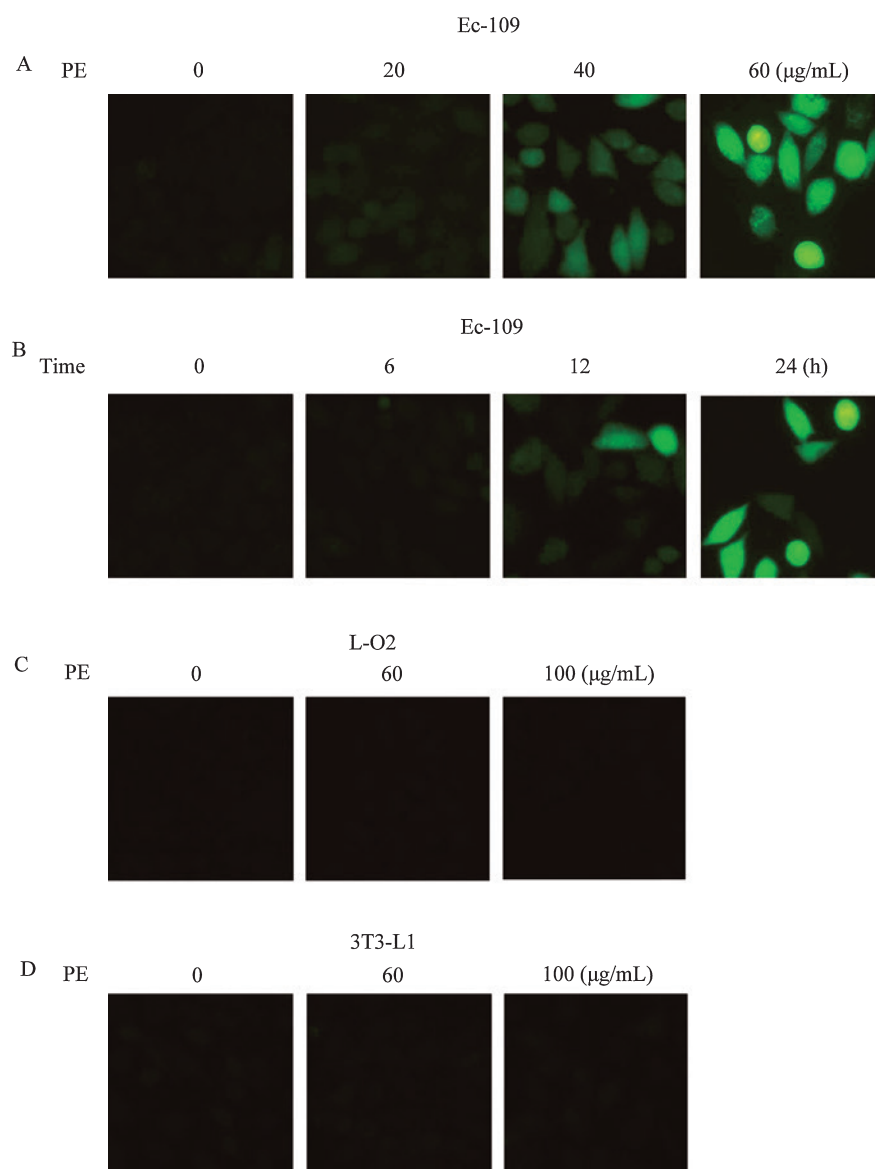


Fig. 4. PE upregulated cellular ROS in Ec-109 cells. A: Ec-109 cells were treated with 0, 20, 40, and 60 $\mu\text{g/mL}$ of PE for 24 h. Then the cellular ROS level was determined using the ROS fluorescence probe DCFH-DA. B: Ec-109 cells were treated with 60 $\mu\text{g/mL}$ of PE for the indicated times. The ROS levels were examined by fluorescence staining as in (A). C and D: L-O2 (C) and 3T3-L1 (D) cells were treated with different concentrations of PE for the indicated times.

ports considering the development of PE as a potential chemotherapeutic reagent for EC treatment.

In multicellular organisms, apoptosis is an evolutionarily conserved biological process of cell suicide required for eliminating useless or potentially harmful cells. It is characterized by morphological alteration of the cells and includes shrinking, chromatin condensation, and formation of apoptotic bodies. Moreover, some changes at the molecular level, including DNA fragmentation and activation of the caspase family of cysteine proteases, occur during apoptosis. It has long been established that disruption of the normal control of apoptosis is implicated in the development of carcinogenesis. Some recent reports demonstrate a very strong association between the genetic variants in the genes involved in the

apoptosis pathway and the risk of EC onset (25 – 26). These findings suggest that inducing EC cell apoptosis by chemotherapeutic agents may be an efficient way for the treatment of this disease. In this study, we revealed that PE induced Ec-109 cell death via a conventional caspase-dependent apoptosis pathway. Up-regulating the cellular ROS level was the key event to trigger Ec-109 cell apoptosis. In aerobic cells, ROS originates as a by-product of oxygen phosphorylation in the electron transport chain within the mitochondria. It is well established that cellular ROS triggers an array of pro-apoptotic molecular events. Many researchers have reported that ROS is involved in chemotherapeutic agent-induced cancer cell apoptosis (27 – 29). Similarly, in our system, PE up-regulated the cellular ROS level in both a time-

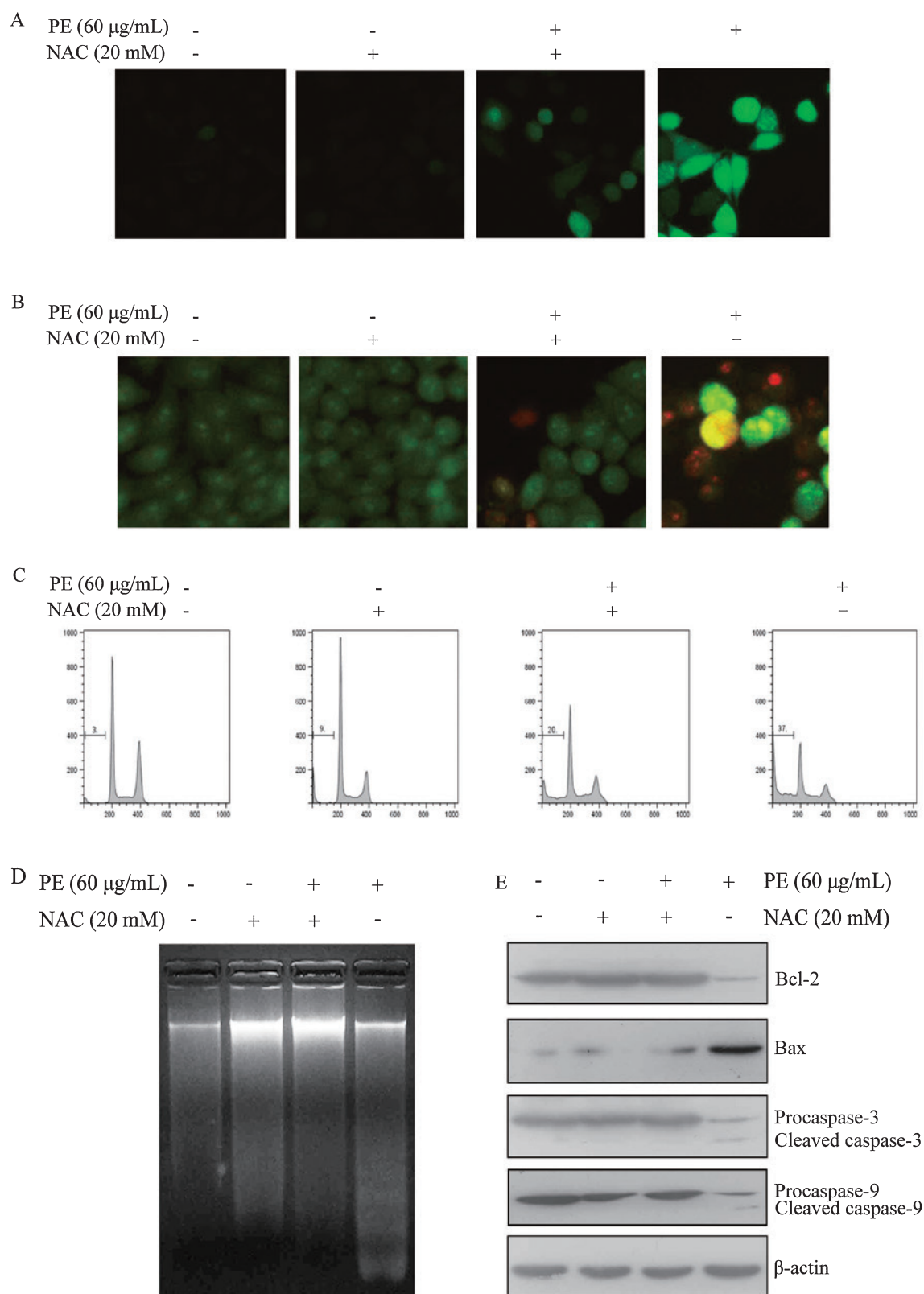


Fig. 5. PE-induced Ec-109 cell apoptosis is mediated by ROS accumulation. A: Ec-109 cells were treated with 60 $\mu\text{g/mL}$ of PE in the presence or absence of 20 mM NAC for 24 h. Then the cellular ROS levels were examined. B, C, and D: Ec-109 cells were treated as indicated for 48 h. Then cell apoptosis was determined by AO/EB staining (B), FACS (C), and DNA fragmentation assay (D). E: After treating Ec-109 cells as indicated for 48 h, the protein levels of Bcl-2, Bax, caspase-3, and caspase-9 were detected by western blotting, using β -actin levels as the loading control.

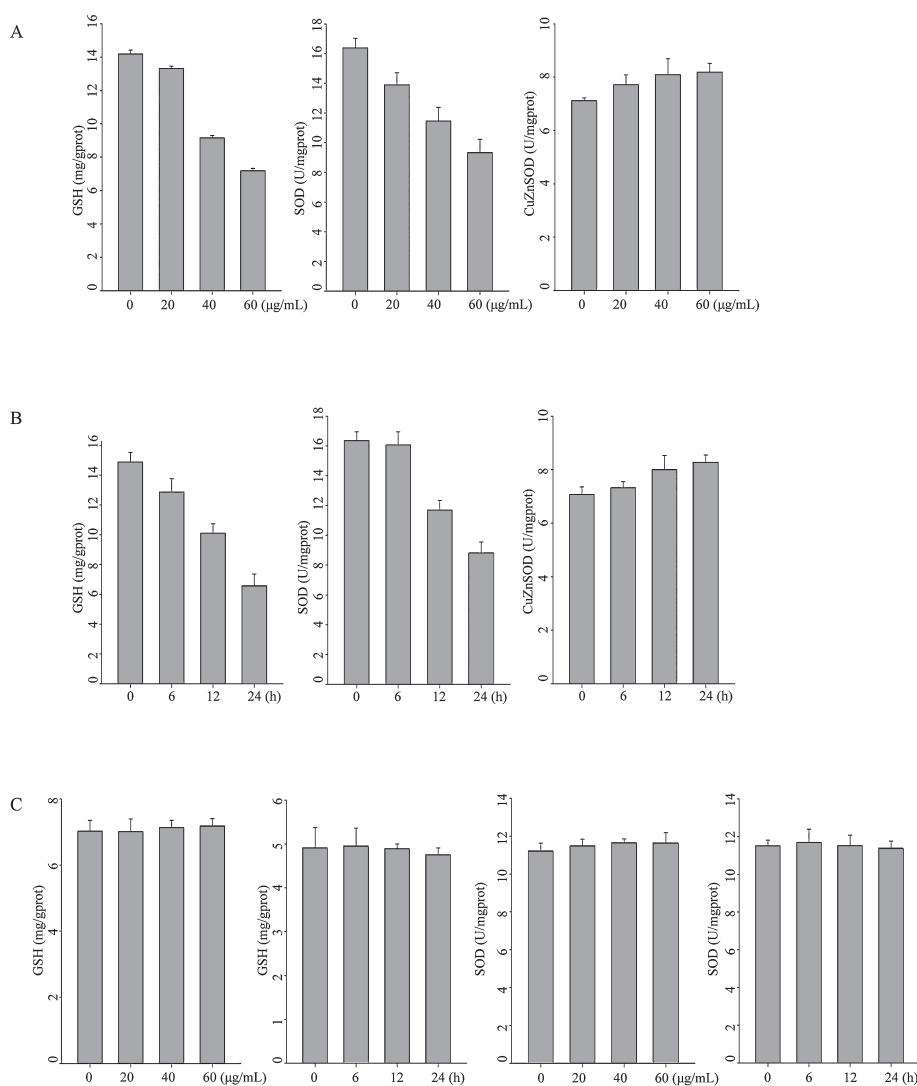


Fig. 6. Effects of PE on GSH, T-SOD, and Cu-Zn SOD levels. A: Ec-109 cells were treated with different concentrations of PE as indicated. After 24 h, cells were subjected to determination of GSH content and SOD activity assay. B and C: Ec-109 (B) and 3T3-L1 (C) cells were treated with 60 µg/mL of PE for different times as indicated. Then, cells were subjected to determination of GSH content and SOD activity assay.

and dose-dependent manner in Ec-109 cells and its apoptosis induction effect was dramatically impaired if the cells were treated with the ROS scavenger NAC. These observations imply the possibility that a low-level of cellular ROS is important for maintaining cancer cell survival and can be disrupted by anti-cancer reagents.

It is well established that cellular ROS is tightly associated with the intrinsic apoptotic pathway, which is modulated by members of the Bcl-2 family of proteins, including both pro- and anti-apoptotic members (30). During apoptosis, the pro-apoptotic proteins in the Bcl-2 family such as Bax translocates to the outer membrane of the mitochondria. This event promotes the release of pro-apoptotic factors, which activate the caspase cascade and induce apoptosis. The anti-apoptotic members in the Bcl-2 family such as Bcl-2 are able to repress this process through interactions with their pro-apoptotic counterparts (31 – 32). In our current study, PE treatment dramatically

decreased Bcl-2 and increased Bax protein levels in Ec-109 cells, a process which was abrogated by the ROS scavenger NAC, and may be responsible for the concomitant execution phase of apoptosis, which included a decrease of $\Delta\psi_m$. Consistently, caspase-9 is the key molecule that modulates the intrinsic apoptotic pathway and its activity could be significantly up-regulated by PE. Additionally, the activity of caspase-8, the key regulator of the extrinsic apoptotic pathway, was not significantly changed during PE treatment (data not shown). Our data suggest that PE induces Ec-109 cell apoptosis via a mechanism involving the ROS intrinsic apoptotic pathway.

Our data showed that in Ec-109 cells, significant cellular ROS accumulation occurred late after PE treatment. This phenomenon is consistent with the fact that there are no oxidative groups in the chemical structure of PE. Therefore, this compound cannot swiftly increase

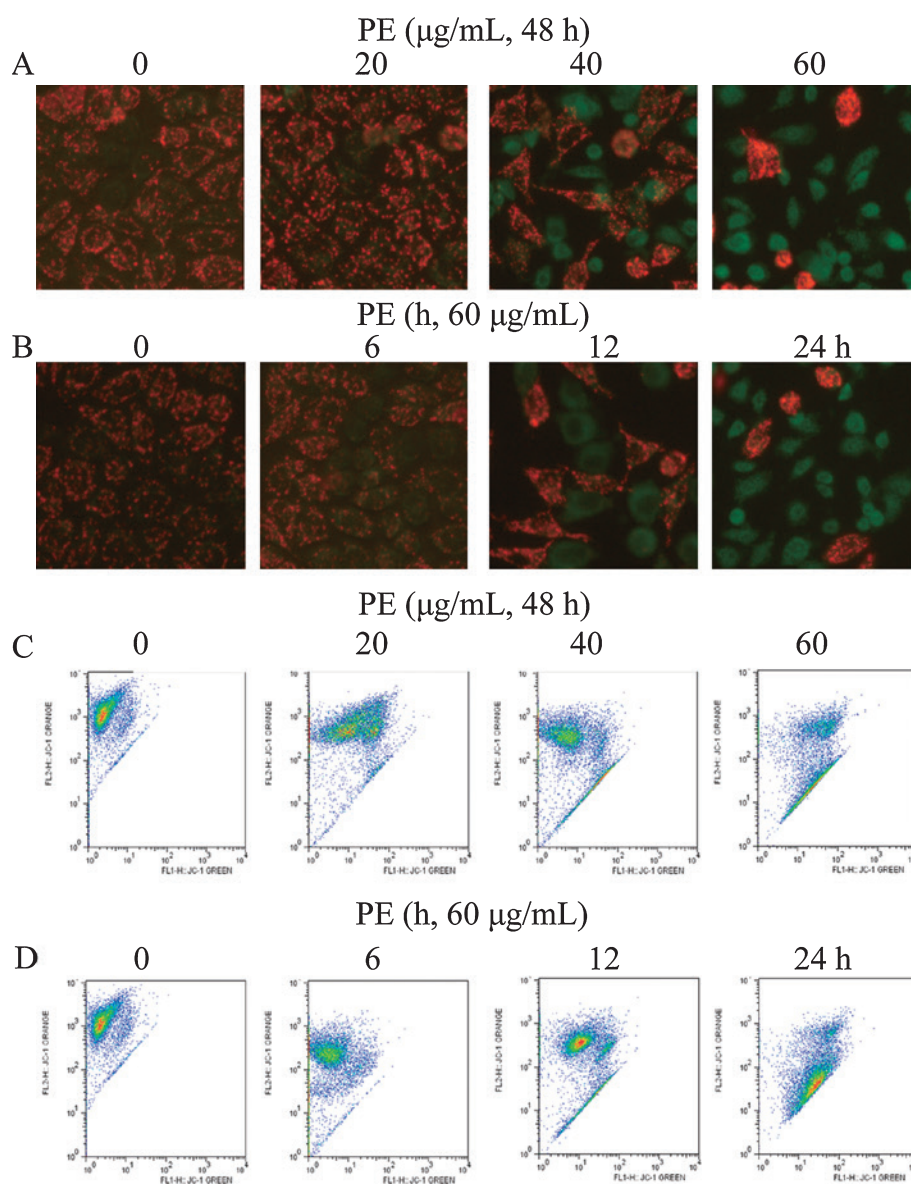


Fig. 7. Effects of PE on mitochondrial membrane potential ($\Delta\psi_m$) in Ec-109 cells. A and C: Ec-109 cells were treated with different concentrations of PE as indicated. After 24 h, cells were subjected to a JC-1 fluorescence staining assay (A) and flow cytometry assay (C). B and D: Ec-109 cells were treated with 60 $\mu\text{g/mL}$ of PE for different times as indicated. Then, cells were subjected to a JC-1 fluorescence staining assay (B) and flow cytometry assay (D).

the cellular ROS level through chemical reactions. It is still not known which proteins are required for PE-mediated ROS-accumulation in Ec-109 cells. Interestingly, in untransformed cells, PE was not able to significantly increase the level of cellular ROS.

ROS scavengers, such as GSH and antioxidant enzymes such as SOD, are the two major components of the cellular anti-oxidant response (33). We found that PE significantly downregulated the levels of cellular GSH and SOD activity in Ec-109 cells. These observations indicated the potential for upstream molecular events that contributed to PE-induced ROS accumulation. It is interesting that the total SOD activity was repressed by PE, but that of Cu-Zn SOD could be upregulated. Considering that there are only two sorts of SOD

(Cu-Zn SOD and Mn-Zn SOD) within mammalian cells, a possible explanation for this observation is that PE selectively repressed the activity of Mn-Zn SOD.

Mitochondria are generally considered to be a potential source of cellular ROS and play a significant role in cell apoptosis (34). Mn-Zn SOD is an antioxidant enzyme localized in the mitochondria and involved in apoptosis events regulated by the Bcl-2 family of proteins such as Bax and Bcl-2 (35–36). It was reported that stable over-expression of Mn-Zn SOD can inhibit cell death or apoptosis in several types of cancer cells (37). In addition, some previous research indicated that the activities of some antioxidant enzymes including SOD were considerably upregulated in cancer tissues as compared to their nontumorous counterparts (38–40).

Consistent with this, we unveiled that SOD activity in Ec-109 cells was higher than that in untransformed 3T3-L1 cells. Our observations implied the possibility that high levels of Mn-Zn SOD were required in Ec-109 cells to protect them from oxidant stress. This protection was sharply reduced when the activity of Mn-Zn SOD was repressed after PE treatment. In untransformed 3T3-L1 cells, the basal levels of GSH content and SOD activity are lower compared to those in Ec-109 cells, indicating that they are experiencing a low level of oxidant stress. Therefore, PE treatment caused few cytotoxic effects in 3T3-L1 cells. In addition, western blotting analysis also showed decreased Bcl-2 expression and increased Bax and caspase-3 expression in Ec-109 cells. These results indicate that down-regulation of Mn-Zn SOD activity and an imbalance in Bcl-2 family protein expression play a critical role in PE-induced Ec-109 cancer cells apoptosis.

In conclusion, our current study described the purification and identification of a new natural triterpenoid saponin (PE) from the traditional herb *E. phaseoloides*. We demonstrated that PE exhibited strong in vitro anticancer activity and Ec-109 esophageal cancer cells were more sensitive to PE treatment compared to cancer cells originating from other tissues. Further investigation revealed the PE-induced caspase-dependent apoptosis in Ec-109 cells and this event was mediated by up-regulation of cellular ROS levels. This process was accompanied by a decrease in the activity of SOD and GSH level and a reduction of $\Delta\psi_m$. In summary, we found that PE induced apoptosis in Ec-109 cells through the ROS-mediated mitochondrial pathway. These data supported the traditional applications of this herb and implied the therapeutic potential of PE against esophageal carcinoma.

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