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Punica granatum (pomegranate) leaves extract induces apoptosis through mitochondrial intrinsic pathway and inhibits migration and invasion in non-small cell lung cancer in vitro

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ABSTRACT

Most conventional treatments on non-small cell lung carcinoma always accompany with awful side effects, and the incidence and mortality rates of this cancer are increasing rapidly worldwide. The objective of this study was to examine the anticancer effects of extract of *Punica granatum* (pomegranate) leaves extract (PLE) on the non-small cell lung carcinoma cell line A549, H1299 and mouse Lewis lung carcinoma cell line LL/2 in vitro, and explore its mechanisms of action. Our results have shown that PLE inhibited cell proliferation in non-small cell lung carcinoma cell line in a concentration- and time-dependent manner. Flow cytometry (FCM) assay showed that PLE affected H1299 cell survival by arresting cell cycle progression in G2/M phase in a dose-dependent manner and inducing apoptosis. Moreover, PLE could also decrease the reactive oxygen species (ROS) and the mitochondrial membrane potential (ΔYm), indicating that PLE may induce apoptosis via mitochondria-mediated apoptotic pathway. Furthermore, PLE blocked H1299 cell migration and invasion, and the reduction of matrix metalloproteinase (MMP) MMP-2 and MMP-9 expression were also observed in vitro. These results suggested that PLE could be an effective and safe chemotherapeutic agent in non-small cell lung carcinoma treatment by inhibiting proliferation, inducing apoptosis, cell cycle arrest and impairing cell migration and invasion.

1. Introduction

Lung cancer is the leading cause of mortality among all the tumors, representing a serious public health problem [1]. Although lung cancer incidence rates are dropping in some areas, the 5-year survival rate remains below 20% [2]. According to statistics, estimated 158,000 deaths caused by lung and bronchus tumors in United States, 2015 [3]. Lung cancer is divided by cell type into non-small-cell lung cancer (NSCLC), which includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma, and small-cell lung cancer (SCLC) [4]. Approximately 85% of lung cancer are NSCLC, which are often diagnosed at an advanced stage and associated with poor prognosis [5]. Nowadays, though targeted therapies (include small-molecule tyrosine kinase inhibitors (TKIs) and mono-clonal antibodies) provide some new options [6], lots of these therapies drugs limited utility owing to preexisting primary or acquired secondary resistance or awful side effects [7]. Therefore the development of new safe and effective therapies is essential.

Phytochemicals are well known as safe and nutrient. Scientific researches on it were last for nearly half century, most about antioxidant activity [8,9]. *Punica granatum*(pomegranate) is commonly known as one of the plants used in traditional herbal

Abbreviations: NSCLC, non-small-cell lung cancer; TKIs, tyrosine kinase inhibitors; PLE, *Punica granatum* (pomegranate) leaves extract; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazoliumbromide; DMSO, dimethyl sulfoxide; PI, propidium iodide; Rh123, 2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester; FBS, fetal bovine serum; FCM, flow cytometry; PBS, phosphate-buffered saline; SD, standard deviation; ΔΨm, mitochondrial transmembrane potential; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; EA, ellagic acid; PC, punicalagin.

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medicine for a long time. Over the last decade, there has been a dramatic increase of interest in pomegranate as a nutritional and medicinal product to treat a wide range of human disorders and maladies [4,10,11]. The overwhelming majority of scientific reports focused on the functions of flowers and juice of the *P. granatum* seeds, such as their preventive and curative role in gastro-mucosal injuries, ethanol- and acetone-induced ulceration, cancer chemoprevention and diabetic oxidative damage [12]. Nevertheless, only a few articles have evaluated the role of leaves of *P. granatum* [13], especially in anticancer aspect.

Hence, our study was aimed at exploring the effects of extracts of *P. granatum* leaves extract (PLE) in non-small cell lung cancer (NSCLC) in vitro. Our results provided that PLE could inhibit cell proliferation, induce apoptosis and suppress migration and invasion in NSCLC cell lines. Considering its safety and nutraceutical properties, these data suggested that PLE might be developed as a novel approach to treat non-small cell lung cancer.

2. Materials and methods

2.1. Cell culture and reagents

The human non-small cell lung cancer cell lines, A549 and H1299, the mouse Lewis lung carcinoma cell lines LL/2, were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell were propagated in DMEM or RPMI 1640 media containing 10% heat-inactivated fetal bovine serum (FBS; Cao Yuan Lv Ye Bio-engineering, Hohhot, China) and 1% antibiotics (penicillin and streptomycin) at 37 °C in 5% CO_2. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester (Rh123) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were from Sigma Chemical Co. (St Louis, MO, USA). The standard substances of ellagic acid (EA) and punicalagin (PC) were purchased from MUST Co. (Chengdu, Sichuan, China). The Annexin V-FITC/PI Apoptosis Detection Kit and Hoechst 33258 were purchased from KeyGen Biotech (Nanjing, China). The primary antibodies against MMP-2 and MMP-9 were purchased from Millipore (Billerica, U.S.A.) and Cell Signaling Technology (Beverly, MA, USA), respectively. And the primary antibodies against β-actin was from ZSGB-BIO Co. (Beijing, China).

2.2. Preparation of PLE

The pomegranate leaves of Tunisia soft-seed pomegranate were provided by Yuzhuang Ecological Green Industry Co., Ltd. (Qianxi County, Guizhou Province, China). The preparation of pomegranate leaves extract was followed by previously described methods [13], with minor modification. The 150 g fresh leaves collected were cut into pieces in size of 0.5 cm and extracted with the combination of 800 mL ethanol and 1200 mL H_2O. Following soaked for 2 h at 60 °C, the crude extracts were filtered and concentrated under vacuum. Then 36 g brown solid was collected. All extracts were dissolved in ethanol and evaporated to dry powder in vacuum before using for determination of anticancer activity finally.

2.3. Quantitative determination of punicalagin and ellagic acid in PLE by HPLC

Waters 1525HPLC system (Waters Corp., Milford, Massachusetts, USA) was used in this experiment. The method and chromatographic condition was performed as described before [14,15]. System control and data analysis was processed by Waters Empower 2 software. The chromatographic separation was conducted on Agilent Zorbax SB-C18 column (5 μm, 4.6 × 250 mm). The mobile phase consisted of deionized-glacial acetic acid (A; 99:1, v/v; pH 3.0) and methanol (B) with a flow rate of 1 mL/min. The gradient program was set as follows: 0–70 min, 5–44% B; 70–80 min, 44% B. The chromatogram was detected at a wave length of 256 nm throughout the assay. The column temperature was retained at 30 °C and the injection volume of each sample and standard solution was 20 μL. The HPLC mobile phase was prepared fresh. Samples and mobile phase were filtered through a 0.45 μm membrane filter and then degassed before injected into the column. We firstly established the standard working curves of ellagic acid and punicalagin. In the next, we detected the samples by HPLC.

2.4. Cell viability and colony formation assay

The cell viability of PLE-treated lung cancer cells was estimated by MTT assay [16,17]. Cells(2000–4000 cells/well) were seeded in 100 μL/well in 96-well plates, and treated with vehicle control (DMSO) or diverse concentrations of PLE after 24 h. After treatment for 24, 48 and 72 h, respectively, 20 μL of MTT solution (5 mg/mL) was added into each well and incubated for an additional 2–4 h at 37 °C in 5% CO_2. The medium was subsequently removed carefully, the purple-colored precipitates of formazan was dissolved with 150 μL of DMSO. The color absorbance was detected at 570 nm by a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, USA). The 72 h MTT assay was also conducted on HEK293 (human embryonic kidney cell line), LO2 (human normal liver cell line) and Vero (African monkey kidney cell line) cell lines to test the cytotoxicity of PLE on normal cells. Colony formation assay [18], cells were seeded in specified number (400–600 cells/well) in 6-well plates, and treated with various concentrations of PLE after 24 h incubation. The fresh medium containing PLE or not was changed every three days. After treatment of 12 days or so, the cells were fixed with methanol and stained with a 0.5% crystal violet solution for 20 min, and the colonies (>50 cells) were counted under microscope.

2.5. Wound-healing migration assay

To evaluate the ability of cell migration, wound-healing migration assay was performed as described previously [19–21]. When cancer cells grew to 80% confluence, cell monolayer was scraped by sterile 0.01 mL pipette tips, and fresh medium containing various concentrations of PLE was added. After 48 h incubation, cells were fixed and photographed. Images were acquired using a microscope (Olympus, IX73, Japan) and the percentage inhibition of migrated cells was expressed using 100% as the value assigned for untreated group.

2.6. Morphological analysis by Hoechst staining

An apoptotic cell presents special morphologic characteristics such as cell body shrinkage, chromatin condensation and margination as well as emerging apoptotic bodies [18]. To identify whether the PLE-induced cell viability reduction was attributable to apoptosis, we stained H1299 and LL/2 cells with Hoechst 33258 dye [20]. Briefly, H1299 and LL/2 cells ($1–2 \times 10^5$ cells/well) were seeded onto 18-mm coverglass in a 6-well plate for 24 h, respectively. After treatment with various concentrations of PLE for following 48 h, cells were washed with cold phosphate-buffer saline (PBS) and fixed in methanol for 15 min. The cells were stained with the Hoechst 33258 solutions according to the manufacturer's instructions. The nuclear morphology of apoptotic cells was observed under a fluorescence microscopy (Olympus, BX53, Japan).

2.7. Cell cycle and apoptosis analysis by flow cytometry (FCM)

To confirm the cell cycle distribution, the H1299 cells were treated with either negative control or different concentrations of PLE for 48 h. Next, the cells were harvested, washed with cold PBS twice, fixed in 70% ethanol (−20 °C) for 2 h and followed by incubated with 0.3 mL of a solution containing 50 μg/mL propidium iodide (PI) for 30 min at 37 °C in the dark, and then analyzed by FCM (BD Biosciences). Aimed at the apoptosis inducing effect of PLE, Annexin V-FITC/PI Apoptosis Detection Kit was used as described before [16]. In brief, cells ($1–2 \times 10^5$ cells/well) were seeded in a 6-well plate for 24 h and then treated with PLE for 48 h. After collected, cells were washed with cold PBS twice. Following the manufacturer's instructions, cells were detected by FCM.

2.8. Mitochondrial membrane potential (Ψ_m) assay and detection of reactive oxygen species (ROS)

Mitochondrial membrane potential assay was conducted as previously reports, determined by FCM using Rh123 staining [22]. Cells, treated with different concentrations of PLE for 48 h, were washed with cold PBS twice and incubated with 5 μg/mL Rh123 at 37 °C in the dark for 30 min. Rh123 fluorescence was then detected by FCM. For the intracellular ROS detection [23], cells were treated with various concentrations of PLE for 48 h and then were harvested and mixed with 10 μM DCFH-DA diluted in PBS at 37 °C for 30 min. Cells were analyzed by FCM.

2.9. Boyden chamber migration and invasion assay

Boyden chamber (8 μm pore size) migration assay was performed according to previously described [16,21], with some modification. In brief, a total of 1×10^5 H1299 cells in 100 μL serum-free medium were added in the upper chamber and 600 μL medium containing 10% FBS was added at the bottom. Different concentrations of PLE were added in both chambers. Cells were allowed to migrate for approximately 48 h. Non-migrated cells in the upper chamber were discarded using a cotton swab. The migrated cells were fixed in methanol and stained with 0.5% crystal violet. Migrated cells in six randomly selected fields were counted and photographed under a light microscope. Invasion assay was conducted as previous studies [16,21]. Briefly, the upper surface of the transwell membrane were coated with serum-free medium diluted Matrigel (1: 3, 60 μL/well, BD Biosciences) and the lower compartment of the chambers were filled with 600 μL medium containing 10% FBS. After Matrigel polymerization, 1×10^5 H1299 cells in 100 μL serum-free medium were added into the upper chamber and treated with various concentrations of PLE. Incubated for 48 h, non-migrated cells on the upper side of the filter were removed, and migrated cells on the underside of the filter were fixed with methanol and stained with 0.5% crystal violet. Then, migrated cells were counted under a light microscope. The result was expressed as the percentage inhibition rate of migration compared with control group.

2.10. Western blot analysis

The western blot analysis was conducted as described previously, with slight change [24]. In brief, H1299 cells were treated with designed concentration of PLE for 48 h, and then cells were washed twice with cold PBS and lysed in RIPA buffer. The protein concentrations were measured by G250 method and equalized before loading. The same amount of protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE) gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, U.S.A.). Next, the membranes were blocked for 1 h at 37 °C and incubated with specific primary antibodies overnight at 4 °C. After incubation with the relevant secondary antibodies and washing the membranes several times, the reactive bands were identified using an enhanced chemiluminescence kit (Millipore, Billerica, U.S. A.).

2.11. Statistical analysis

Data are represented as mean ± standard deviation (SD) of three independent experiments. The two-tailed Student's *t*-test was used for statistical analysis, and *P*-values were labeled as follows: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. The content of ellagic acid and punicalagin in PLE

Linear regression analysis for EA and PC was expressed by plotting the peak area (y) against the concentrations (x, mg/L) of standard solutions, as shown in Table 1. All the analytes presented good linearity in the investigated ranges. The sample of PLE was analyzed using the optimized HPLC method and matched by *the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine* (Version 2004A). Some components were identified as punicalagin (peak 1) and ellagic acid (peak 2) by comparing the retention time of standard substances, which was shown in Fig. 1A and B. According the regression equation and the relation peak area of PLE, the content of punicalagin and ellagic acid in PLE were calculated. The content of punicalagin and ellagic acid are 39.6 mg/g and 32 mg/g in PLE, respectively.

3.2. PLE inhibits NSCLC cells proliferation and the morphology and has little cytotoxicity on normal cells

MTT assay was conducted to determinate the performance of PLE on the proliferation of human NSCLC A549 and H1299 cells and Lewis lung cancer LL/2 cells. These cells were treated with PLE at the concentration of 0, 6.25, 12.5, 25, 50, 100, 200 μg/mL for 24, 48 and 72 h, respectively. As revealed in Fig. 2A, PLE inhibited the lung cancer viability in concentration- and time-dependent manner. The results of MTT assay on HEK293, LO2 and Vero presented that PLE showed no obvious cytotoxicity on normal cells (Table 2). Moreover, PLE has a high selectivity index (more than 10 times), indicating that PLE has a better security.

To further investigate whether PLE could inhibit viability of NSCLC cells, we conducted clonogenic assay after PLE treatment. As shown in Fig. 2B, clonogenic assay crisply expressed that clone formation of A549, H1299 and LL/2 cells was reduced in concentration-dependent manner after exposure to PLE. However, the concentration of 6.25 and 12.5 μg/mL group didn't show obviously compared to the control. These results were consistent with the MTT data. Taken together, those results implied that PLE had a strong cytostatic and cytotoxic effect in NSCLC cells.

Table 1
Linear range, regression equation and R^2 of punicalagin and ellagic acid.

Component	Linear range (mg/L)	Regression equation	R^2
EA	50–500	[a] $y = 149530x + 2 \times 10^6$	0.9935
PC	125–2000	[a] $y = 2 \times 10^7 x - 6 \times 10^6$	0.971

[a] y is the peak area; x refers to the concentration of compound (mg/L).

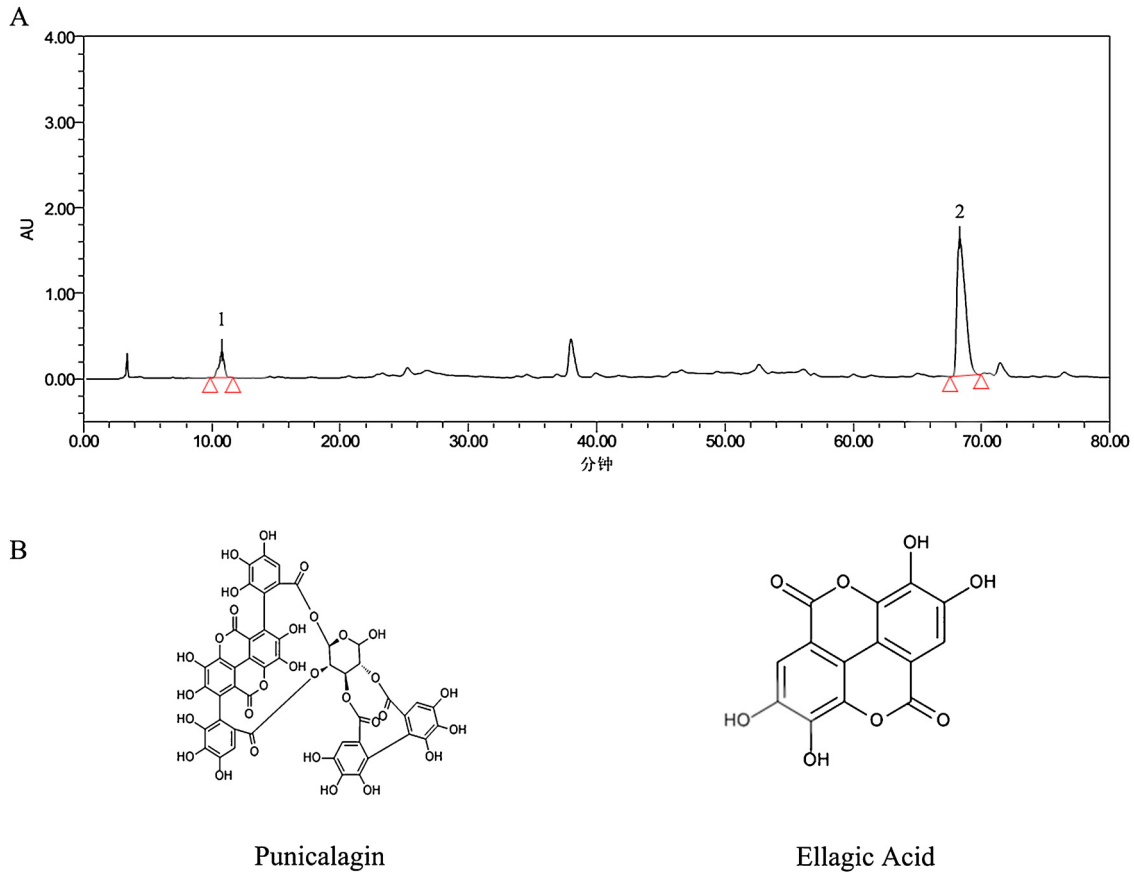

Fig. 1. HPLC fingerprint of PLE (A) and structures of two main contents in PLE, punicalagin and ellagic acid (B). HPLC conditions-column: Agilent Zorbax SB-C18 column (5 μm, 4.6 ± 250 mm); mobile phase: (A) deionized-glacial acetic acid (99:1, v/v, pH 3.0), (B) methanol; gradient program: 5–44% B (0–70 min), 44–44% (70–80 min); flow rate: 1.0 mL/min; detection wavelength: 280 nm; temperature: 30 °C; injection volume: 20 μL.

3.2. PLE induced cell apoptosis and cell cycle abnormalities in H1299 cells

We next explored whether PLE induced lung cancer cells apoptosis. Hoechst 33258 staining assay showed that PLE treated induced apoptosis in H1299 and LL/2 cells, with the features of a brightblue flourencent-condensend nuclei and nuclear fragmentation (Fig. 2C). To further confirm the induction of apoptosis in H1299 cells with PLE treatment, we also investigated the levels of apoptosis by low cytometry (FCM) using the Annexin V-FITC/PI dual-labeling technique. As shown in Fig. 4A and B, after PLE treatment for 48 h, the apoptosis induction effects were apparently observed. Compared to the control group, the PLE groups showed significant apoptosis. When the H1299 cells were treated with 12.5 μg/mL of PLE, the apoptosis rate was 8.61%, whereas the apoptosis cells increased to 44.1% when cells were treated with 200 μg/mL PLE, respectively, indicating that PLE was able to induce apoptosis in a relatively high concentration.

To illustrate the molecular mechanisms underlying the anticancer effects of PLE, we evaluated its potential effect on cell cycles distribution by FCM analysis in H1299 cells. As shown in Fig. 3A, the cell cycle was disrupted significantly after the treatment with PLE for 48 h, especially in the dose of 100 μg/mL. A statistics of the cell population in G0/G1, S and G2/M phases of the cell cycle was presented in Fig. 3B. These results demonstrated that PLE could block the H1299 cell cycle progression in G2/M phase with increasing dose.

3.3. PLE induced loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) and ROS

In order to identification that PLE induces the disruption of the mitochondrial membrane potential, we used a mitochondria specific and voltage-dependent dye Rh123 to detect alterations in $\Delta\Psi_m$ in H1299 cells. As shown in Fig. 5B and C, PLE treatment led the loss of $\Delta\Psi_m$ potential in H1299 cells, especially in the dose of 100 μg/mL. These results suggested that the mitochondria-mediated pathway may be involved in PLE-induced apoptosis.

The ROS accumulation can change the intracellular environment [25], and induce apoptosis further. For detection of ROS, we stained the cells with DCFH-DA reagent and estimated ROS accumulation by FCM. We discovered that the treatment of PLE lessen the ROS accumulation in a dose-dependent manner, which can be observed in Fig. 5A explicitly.

3.4. PLE impaired NSCLC cells migration and invasion

Lung carcinoma metastasis poses a foremost threat to cancer related mortality. Moreover, one of the key steps in successful cancer metastasis is tumor cell migration and invasion [26]. Hence, it is imperative to investigate whether PLE could inhibit lung

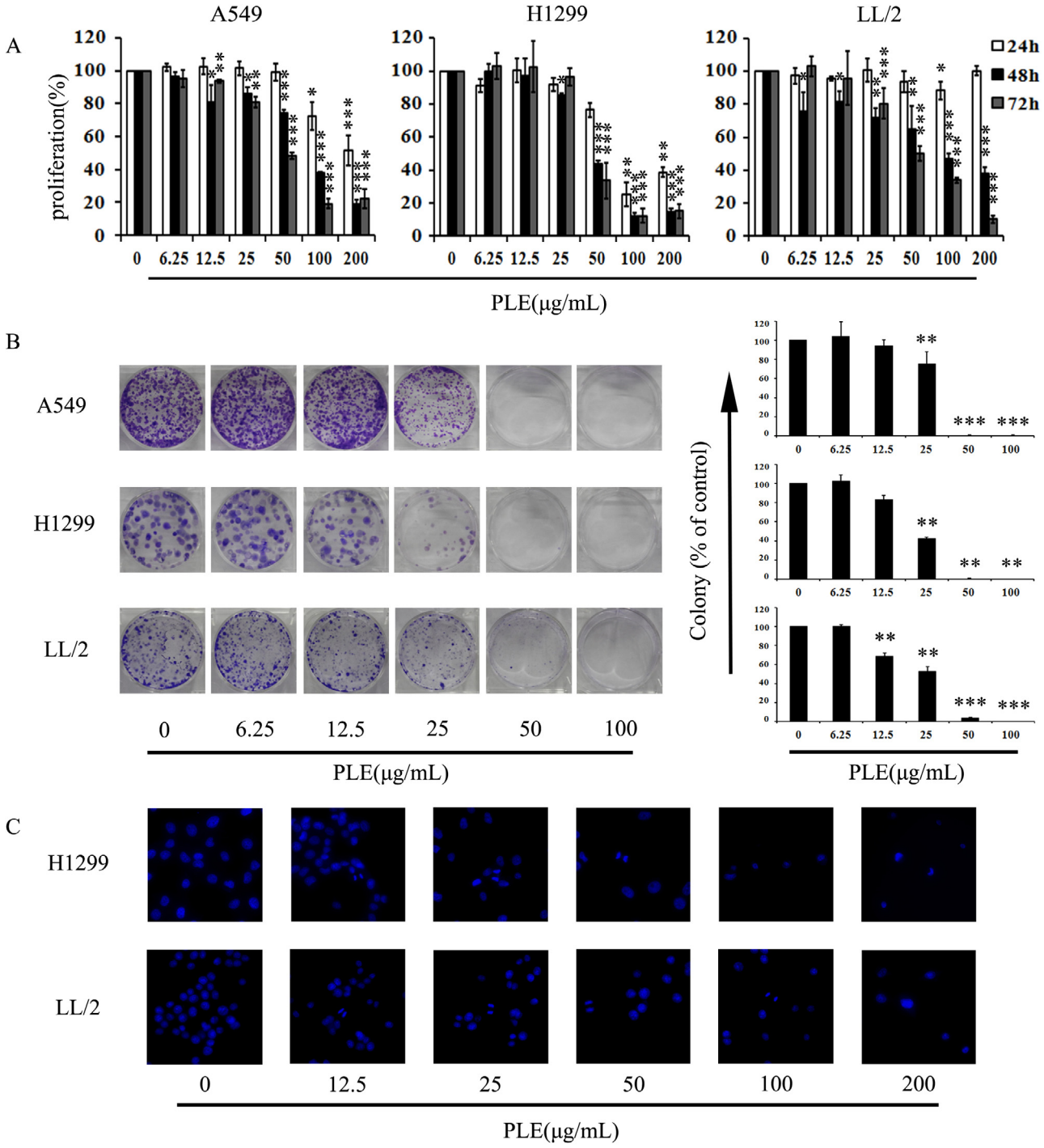

Fig. 2. The effect of PLE on non-small lung cancer cells viability. (A) Proliferation of A549, H1299and LL/2 cells treated with various concentrations (0–200 μg/mL) of PLE for 24, 48 and 72 h, respectively. Cell viability was detected by MTT assay. (B) The effects of PLE (0–200 μg/mL) on colony formation in A549, H1299 and LL/2 cells for about 12 days, the statistic results of colony formation assays presented as surviving colonies. Data are expressed as means ± SD from three experiments ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (C) The fluorescence microscopic appearance of Hoechst 33258 staining nuclei of H1299 and LL/2 cells with various concentrations of PLE for 48 h. Data are the representative from three parallel experiments.

cancer cell migration and invasion, we conducted wound-healing and transwell migration assays using H1299 cells. As was shown in Fig. 6A, PLE inhibits migration of H1299 and LL/2 cells in a dose-dependent manner. Similar results were obtained in transwell migration assay (Fig. 6B). Then we performed a Matrigel invasion assay. Fig. 6C showed that H1299 cells decreased invasion significantly in the exposure of PLE than vehicle control, which was in a dose-dependent manner. As Fig. 7 illustrated, the treatment of PLE could decrease the expression of MMP-2 and MMP-9, which are considered to play significant roles in cell migration and invasion. From the above, our results expressed that PLE possessed a strong ability on NSCLC cells migration and invasion.

4. Discussion and conclusion

Non-small-cell lung carcinoma is one of the most serious diseases and accounting for almost 85 percent of lung cancer.

Table 2
The effects of PLE in NSCLC cells and normal cell lines viability.

Cell line	Cell type	IC_{50} (μg/mL)
A549	Human non-small lung carcinoma cell line	50
H1299	Human non-small lung carcinoma cell line	47
LL/2	C57BL Lewis lung carcinoma cell line	54
HEK293	Human embryonic kidney cell line	>600
LO2	Human normal liver cell line	>600
Vero	African monkey kidney cell line	>600

MTT assay was employed to detect the cell viability. Each cell line was treated with various concentrations of PLE for 72 h, respectively. Data are expressed as the mean from three experiments.

Nowadays, treating this disease is still given priority to surgery clinically. Platinum-based drugs and taxol, taxotere and other new anticancer drugs are used extensively for chemotherapy [27]. The side effects are awful and most tumor cells become resistant to drug [28,29]. In addition, Despite targeted NSCLC the therapeutics could increase survival time and quality of life for cancer patients, have limited utility owing to preexisting primary or acquired secondary resistance or side effects [7]. Therefore, the search of effective clinical approaches for the treatments of this cancer is still a challenge. Herbal plants have been the basis for almost all medicinal therapies and remain one of the most popular

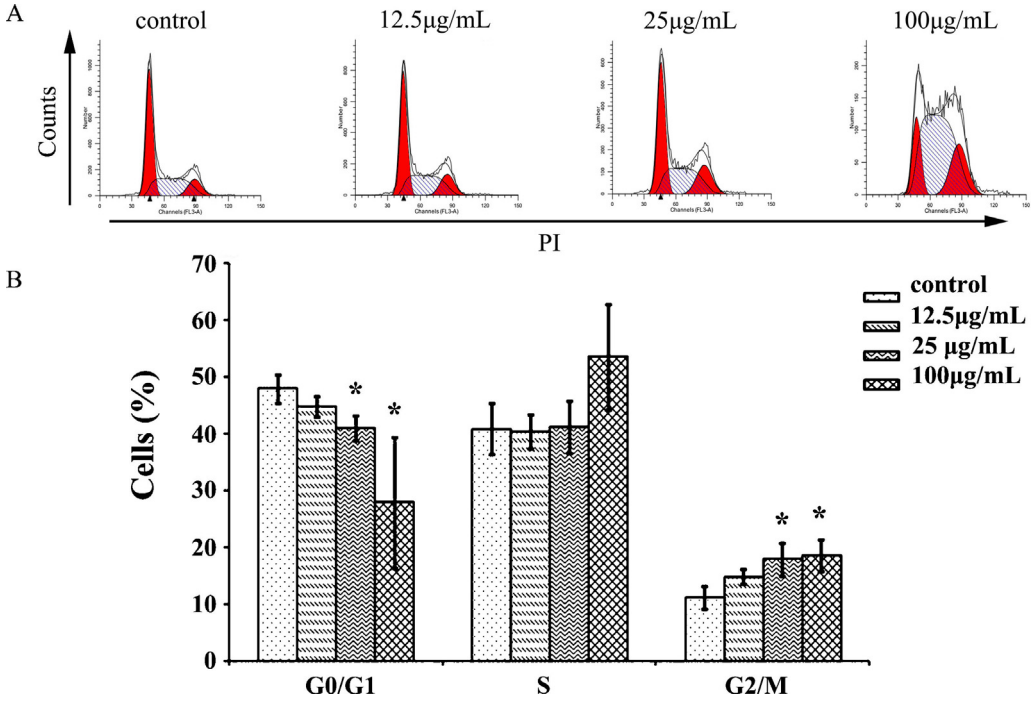

Fig. 3. Effects of PLE on cell cycle progression of H1299 cells. (A) H1299 cells were exposed to various concentrations of PLE (0–100 μg/mL) for 48 h followed by analysis of cell cycle by flow cytometry. (B) Distribution of H1299 cells at different phases of the cell cycle distribution. $^{*}p < 0.05$ compared to control.

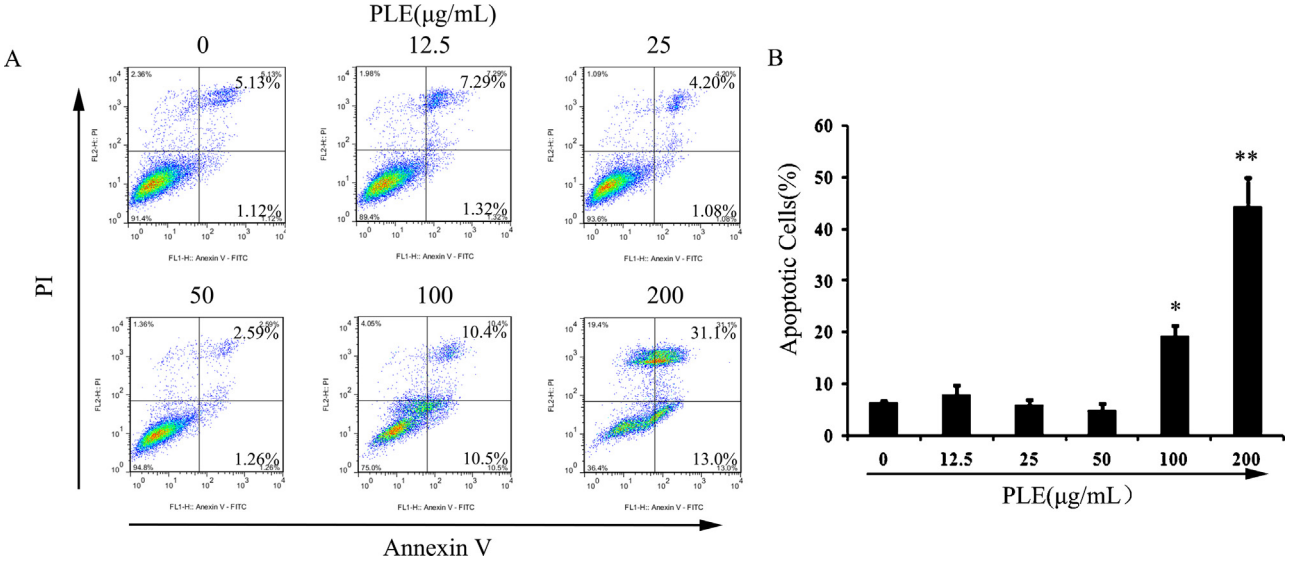

Fig. 4. Induction of apoptosis in H1299 cells by PLE treatment. (A) H1299 cells were treated with PLE at indicated doses for 48 h. (B) The apoptosis rate was treated statistically. Data are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

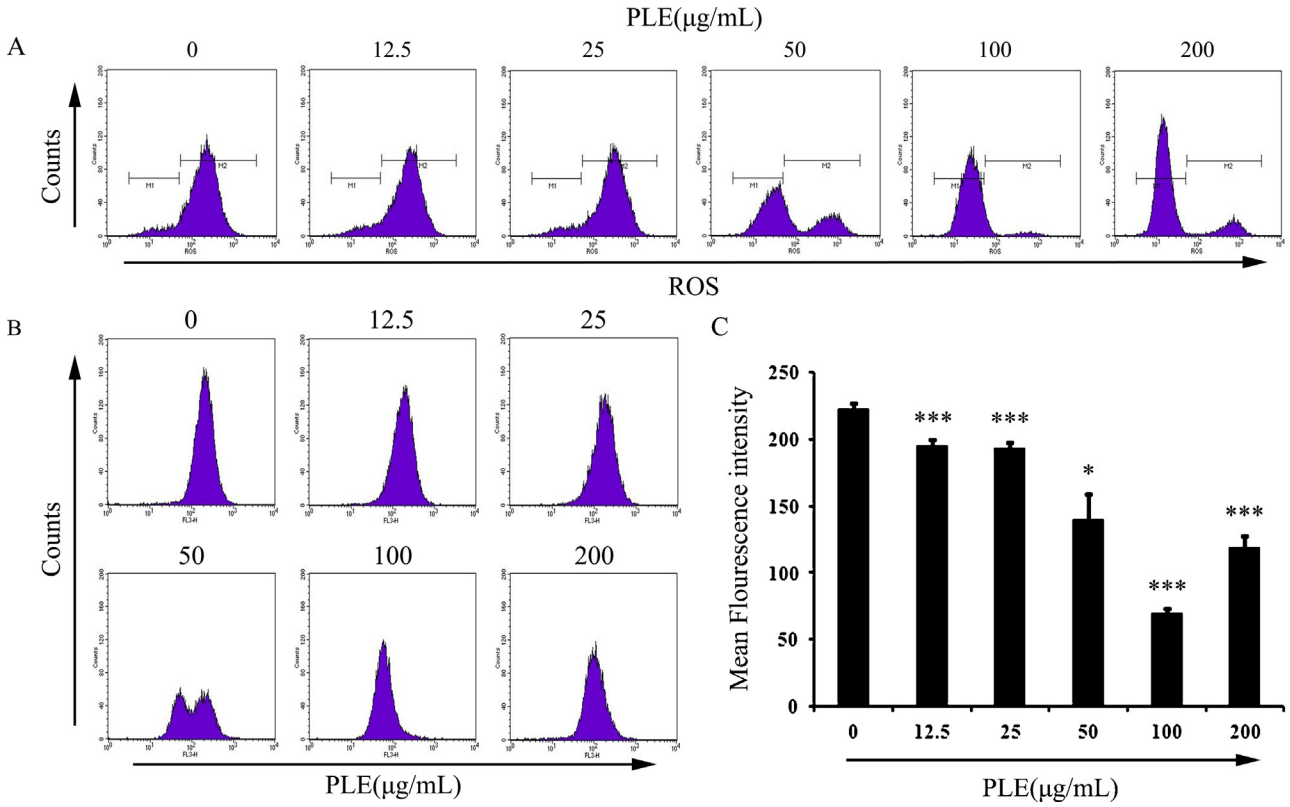

Fig. 5. The change in the mitochondrial membrane potential ($\Delta\Psi_m$) and the accumulation of ROS. (A) H1299 cells were treated with PLE at indicated doses for 48 h followed by analysis of ROS by flow cytometry. (B) H1299 cells were exposed to various concentrations of PLE (0–200 μg/mL) for 48 h. (C) The mean fluorescence intensity detected in the mitochondrial membrane potential assay was treated statistically. Data are expressed as mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to control.

alternatives for cancer prevention and treatment all over the world for a long time [12]. Recently, there has been a growing interest in investigating the effects of phytochemicals on the inhibition of cancer cell growth in combination with chemotherapeutics or other therapies [30–33], such as curcumin from *Curcuma longa*, epicatechin gallate from tea and paclitaxel from Pacific yew. Because of its safety and nutrition, phytochemicals would be a promising strategy to provide complementary approaches for cancer prevention and treatment. Currently, there are lots of studies on most pomegranate (*P. granatum* Linn., Punicaceae) parts which are known to possess enormous antioxidant and antibacterial activity. Therefore, our report focused on the anticancer effects on NSCLC of the extract of pomegranate's leaves (PLE).

In this study, we firstly detected the content of EA and PC in PLE, which were 32 mg/g and 39.6 mg/g. These polyphenols constituted the main composition of extract and played an important role in anticancer activity [4,34,35]. The accumulation of EA was superior to previous report, while that of PC was inferior [14]. In this study, we provide evidence that PLE decreased proliferation and viability of A549, H1299 and LL/2 cells in various aspects, covering induction of apoptosis and arrest of cell cycle. MTT assay demonstrated that PLE could inhibit the growth and proliferation of A549, H1299 and LL/2 carcinoma cells in a dose- and time-dependent manner. Through the pairwise contrast, we discover that cells explored to PLE 0, 6.25, 12.5, 25, 50 μg/mL for 24 h, had no statistically apparent difference. In other words, PLE had little toxic activity on cancer cells at low dose for a short time. Meanwhile, MTT assay on normal cells, including HEK 293, LO2 and Vero, indicated that PLE had no or little toxicity on normal cells (selective index more than 10 times). In other word, PLE could be a safe and efficient medicine for NSCLC. As was shown in Fig. 2B, compared to the control, classic apoptotic morphologic changes which include reduction of cell volume and formation of a circular morphology were observed in PLE-treated cells. These results were in keeping with MTT assay.

Apoptosis is a consequence of a highly complex and sequential cascade of cellular events and essential for the development and maintenance of tissue homeostasis [31]. Therefore, inducing apoptosis is a therapeutic method to arrest cancer progression [12,36]. In our study, the results expressed that PLE induced apoptosis of H1299 cells, especially in the dose of 100 and 200 μg/mL. Moreover, Measurement of mitochondrial membrane potential study results demonstrated that PLE significantly reduced mitochondrial membrane potential ($\Delta\Psi$m) of H1299 cells. Furthermore, ROS plays an important role in the course of apoptosis in many cell types, in cancer cells, ROS production significantly contributed to apoptosis triggering [37]. It would destroy intracellular homeostasis whether the ROS accumulation up or down [38–40]. Thus, we also evaluated the accumulation of ROS. When cells were treated with PLE for 48 h, the ROS accumulation decreased in a dose-dependent manner. Taking together, these results might explain the finally apoptotic death of H1299 cells by PLE, at least in part, the results of present study could also reveal that PLE induced death by mitochondrial mediated apoptosis pathway.

Cell cycle arrest is the main way to inhibit tumor cell proliferation, and dephosphorylation of AKT in the process of decreased ROS levels activating the inhibition of cell proliferation plays an important role [38,41,42]. The ROS might induce DNA damage, and then DNA damage could result cell cycle arrest [43]. Activation of cell cycle checkpoints, which includes the G1-S or/and G2-M transition, is a general cellular response following

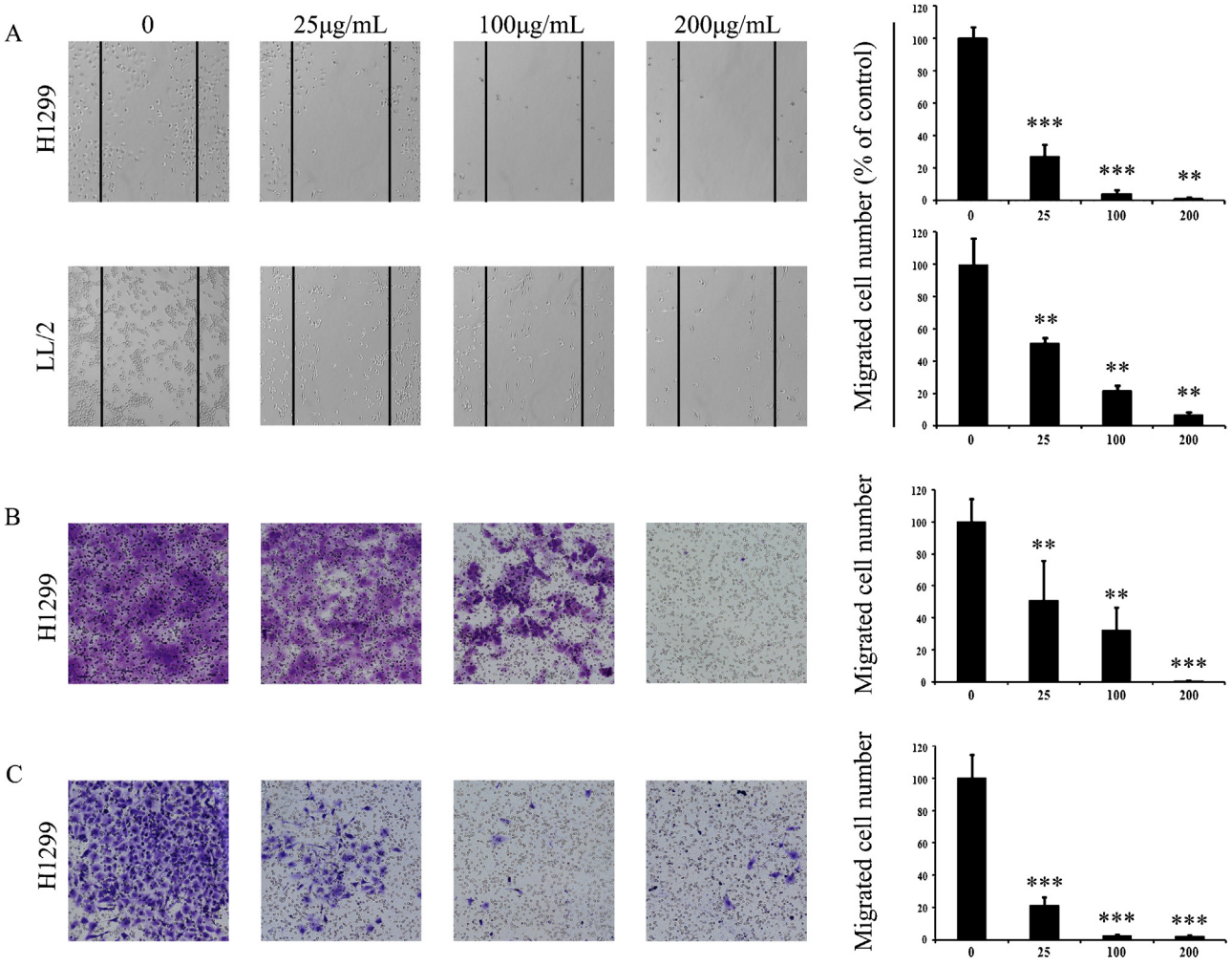

Fig. 6. Inhibition of migratory and invasive of PLE-treated H1299 cells. (A) H1299 and LL/2 cells were seeded in six-well plates. A single scratch was made after the cells grew about 80% confluence. After treatment of PLE for 48 h, the groups were fixed and photographed. The lines indicate the area occupied by the initial scraping and migrated cells were quantified. (B) H1299 cells were seeded in the top chamber of transwell with serum-free medium and treated with vehicle or various concentrations of PLE. After about 48 h, migrated cells were fixed, stained, and photographed and quantified. (C) H1299 cells were treated with different concentrations of PLE and allowed to invade through Matrigel. Invaded cell number was counted. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control.

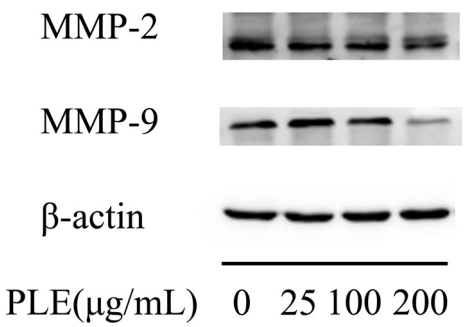

Fig. 7. The change of MMP-2 and MMP-9 expression. H1299 cells were treated with different concentrations of PLE. After 48 h, cells were harvested, and western blot assay was conducted to test the expression of MMP-2 and MMP-9. β-actin served as loading control.

exposure to cytotoxic agents [44,45]. In our study, we found that cytotoxicity of PLE might arrest H1299 cell cycle progression in G2/M phase with increasing dose of PLE.

NSCLC has a high rate of metastasis to distant sites such as brain, spleen and bone [46]. When the NSCLC is examined out, it always has been accompanied with metastasis. During tumor metastasis, cancer cell migration and invasion is a critical step and suppression of the step is a meaningful approach to anticancer treatment [26]. H1299 cells have a highly migratory ability [47,48]. Recent studies have reported that the level of MMPs have established a positive relation with cancer cell migration and invasion [19,48]. Herein, we took assays to support the anti-metastasis ability of PLE in vitro by targeting cancer cell migration and invasion. In this study, PLE down-regulated MMP-2 and MMP-9 expression in H1299. Therefore, we discovered that treatment of metastatic NSCLC cell line (H1299) with PLE significantly restrained tumor cell migration and invasion.

In summary, PLE can suppress the proliferation of NSCLC cells in vitro and can induce cell apoptosis through mitochondrial pathway associated with loss of mitochondrial transmembrane potential ($\Delta\Psi$m) and the down regulation of ROS accumulation. What's more, PLE could arrest cell cycle in G2/M phase which also explained effects of PLE in NSCLC cells. Additionally, PLE impaired NSCLC cell migration and invasion. In all, above mechanisms might present a better understanding for cytotoxicity of PLE and promote its potential use as a safe and effective therapeutic agent in NSCLC.

Conflict of interest

The authors declare that they have no competing interests.

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