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Original article

The extract from *Punica granatum* (pomegranate) peel induces apoptosis and impairs metastasis in prostate cancer cells

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ABSTRACT

Prostate cancer is a big threat to male for its poor prognosis and high mortality rate. Natural compounds are important resources of many anticancer drugs. Pomegranate is a kind of antioxidant-rich fruit and its peel and seed has potential anticancer activities. In this study, we aimed to investigate the effects of pomegranate peel extract (PoPx) on the apoptosis and metastasis of prostate cancer cells and the related mechanism. We found that PoPx showed growth inhibition on prostate cancer cells. Nuclei morphological and flow cytometer (FCM) analysis indicated that PoPx could induce prostate cancer apoptosis. Further investigation indicated that mitochondrial mediated intrinsic pathway is involved in the apoptosis. Exposure to PoPx led to loss of mitochondrial transmembrane potential (Δym), accumulation of reactive oxygen species (ROS). Western blot analysis showed that PoPx could increase the expression ratio of Bax/Bcl2 and activation of apoptosis executor caspase 3. Wound healing assay and transwell migration and invasion assay implied that PoPx has the potential to inhibit migration and invasion, two critical steps in prostate cancer metastasis. Downregulation of MMP2/MMP9 and upregulation of TIMP2 showed accordance with the inhibition of migration and invasion. In summary, the present data showed that PoPx could be a promising drug candidate to treat prostate cancer, showing us a better way to develop novel drugs from natural compounds.

1. Introduction

Cancer is the leading cause of death in the 21st century. According to American Cancer Society, prostate cancer is the most common form of cancer in male, accounting for 1 in 5 new cancer diagnoses in men and ranking the second leading cause of cancer related mortality [1]. Unfortunately, the majority of prostate cancer patients are diagnosed at a late stage with distant metastasis but its pathogenesis is not fully understood yet, resulting a high mortality [2,3]. Thus, there is an urgent need to uncover the molecular mechanisms of prostate cancer (PCa) and to develop some novel and better therapeutic approaches.

Punica granatum (Pomegranate) is one member of Punicaceae. The peel juice and seed oil of the pomegranate fruit is an important source of antioxidants with known anti-cancer activities [4]. Numerous current researches have demonstrated that antioxidants in some fruits and vegetables can defend human cells from oxidative damage and prevent aging, resulting in a decreased incidence of tumors and infections [5]. There are plenty of bioactive components presented in pomegranate peel, such as tannins, flavonoids, polyphenols, anthocyanins, *etc.* [4]. They have been proposed to have various pharmacological activities, including anticancer activity.

Evasion of apoptosis is one hallmark of maybe all types of cancer [6]. Deregulation of apoptosis resulted in uncontrolled

Abbreviations: PCa, prostate cancer; PoPx, *Punica granatum* (pomegranate) peel extract; MTT, 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazoliumbromide; DMSO, dimethyl sulfoxide; 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; $\Delta\psi_m$, mitochondrial transmembrane potential; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MMP-9, matrix metalloproteinase-9; MMP-2, matrix metalloproteinase-2 TIMP2 tissue inhibitors of metalloproteinase 2; CC-3, cleaved-caspase-3; EA, ellagic acid; PC, punicalagin.

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growth of cancer and development of resistance to treatment. Therefore, drugs that could restore cancer cell apoptosis may be effective against many types of cancer, making this a promising strategy to treat cancer [7]. On April 11, 2016, the U. S. Food and Drug Administration approved Venclexta for treating chronic lymphocytic leukemia (CML) patients. This is a Bcl2 inhibitor that specifically targeted to the apoptosis pathway.

It has been proved in earlier studies that pomegranate peel could inhibit prostate cancer growth by inducing cell apoptosis [8]. However, the mechanism of pomegranate peel induced apoptosis has not been delineated clearly yet. Metastasis is the main cause of PCa related mortality. Migration and invasion are two critical steps in metastasis. Numerous proteins are involved in the metastasis cascades. Among them, matrix metalloproteinases could destruct the integraty of extracellular matrix in the microenvironment to facilitate metastasis [9,10]. It's still unknown whether pomegranate peel extract (PoPx) could inhibit PCa metastasis cascades. Hence, this study is aimed to investigate the effects of PoPx on PCa cells and provide evidence for the further application of PoPx in inhibiting PCa growth and metastasis. The effects of PoPx were assessed on several PCa cells and the preliminary molecular mechanism was explored in our study.

2. Materials and methods

2.1. Preparation of pomegranate peel extract (PoPx)

Pomegranate peel of Tunisia soft-seed pomegranate was purchased from Yuzhuang Ecological Green Industry Co. (Qianxi County, Guizhou Province, China). The preparation of pomegranate peel extract was done as previously described with minor modifications. Firstly, 400 g fresh peel was cut into pieces of 0.5 cm in size and extracted with a combination of 1550 mL ethanol and 480 mL H_2O followed by soaking for 2 h at 60 °C. The crude extracts were then filtered and concentrated under vacuum to produce a total of 45 g brown powder. This PoPx was stored at −20 °C and protected from light before use.

2.2. Quantitative analysis of PoPx by HPLC and MS

A Waters 2695 HPLC system (Waters Corp., Milford, Massachusetts, U.S.A.) was used in this experiment. The method and chromatographic conditions was followed by previously described methods. Data were processed by Waters Empower 3 software. The chromatographic separation was conducted on a Waters Symmetry C18 column (5 μm, 4.6 × 250 mm). The mobile phase is composed of deionized water with glacial acetic acid (A, 99:1, v/v, pH 3.0) and methanol (B) with a flow rate of 1 mL min^{-1}. The gradient program was set as follows: 0–70 min, 10–45% B; 70–80 min, 45% B. The chromatogram was detected at a wavelength of 256 nm throughout the assay. An Absciex Qtrap 5500 was used in the MS. We detected the samples by HPLC and MS after establishing the standard working curves of ellagic acid (EA) and punicalagin (PC).

2.3. Reagents and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (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 purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Hoechst 33258 was purchased from Beyotime (Beijing, China). Annexin V-FITC Apoptosis Detection Kit was purchased from KeyGen Biotech (Nanjing, China). For western blot experiments, the primary antibodies against matrix metalloproteinase-9(MMP-9), matrix metalloproteinase-2(MMP-2), TIMP2, Bax, Bcl2, Cleaved-caspase-3 and β-actin were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.) and secondary antibodies were purchased from ZSGB-BIO Co. (Beijing, China). For *in vitro* assays, PoPx was prepared as a 200 mg/mL stock solution in DMSO and stored at −20 °C. Then the stock solution was diluted in the relevant medium and 0.1% DMSO served as vehicle control.

2.4. Cell culture

Human prostate cancer cell lines DU145, PC3, mouse prostate cancer cell TRAMP-C1 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPIM1640 or DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 at 37 °C.

2.5. Cell viability assay

The cell viability of PoPx-treated cancer cells were tested by MTT assay [11]. Firstly, cancer cells in the logarithmic phase were seeded in 96-well plates (100 μL) at 2–6 × 10^3 cells per well for 24 h. After attachment, the cells were exposed to various concentrations of PoPx (0, 12.5, 25, 50, 100, 200 μg/mL) for 24 h, 48 h and 72 h, respectively. Then 20 μL of 5 mg/mL MTT solution was added into each well and incubated for an additional 2–4 h at 37 °C. The liquid waste is discarded and the purple-colored precipitates of formazan were solubilized by the addition of 150 μL DMSO to each well. Finally, the color absorbance was measured at 570 nm by a Spectra MAX M5 microplate spectrophotometer (Molecular Devices, CA, U.S.A.).

2.6. Colony formation assay

Colony formation assay was measured as previously described [12]. Briefly, TRAMP-C1 DU145 and PC3 cells were seeded in 6-well plates at a density of 700–1000 cells per well for 24 h. Then the cells were treated with designated concentrations of PoPx (0, 6.25, 12.5, 25, 50 and 100 μg/mL) and incubated for an additional 710 days. Subsequently, the cells were washed with PBS followed by fixed with methanol and stained with a 0.5% crystal violet solution for about 15 min and the colonies (>50 cells) were counted under a microscope.

2.7. Morphological analysis of nuclei by Hoechst staining

Cells have distinct morphologic characteristics when in the apoptotic process: cell body shrinkage, chromatin condensation and apoptotic bodies. To investigate whether PoPx induced inhibition of in cell viability was attributed to apoptosis, we stained TRAMP-C1 and PC3 cells with Hoechst 33258 dye after PoPx treatment [13]. Briefly, cells at logarithmic growth were seeded onto an 18 mm coverglass in a 6-well plate at a density of 5–10 × 10^4 per well. Twenty-four hours after seeding, the cells were treated with different concentrations (0, 12.5,25, 50, 100, 200 μg/mL) of PoPx for 48 h. Then the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in methanol for 15 min. Then the cells were stained with Hoechst 33258 solution in accordance with the manufacturer's instructions and the nuclear morphology of the cells was photographed under a fluorescence microscope (Leica, DM4000B).

2.8. Apoptosis analysis by flow cytometry (FCM)

An Annexin V-FITC apoptosis detection kit was used to further confirm PoPx induced apoptosis as described before [13]. In brief,

TRAMP-C1 cells ($1–2 \times 10^5$ cells per well) were seeded in 6-well plates. Twenty-four hours later, the cells were treated with PoPx (0, 12.5, 25, 50, 100, 200 μg/mL) for 48 h. Then the cell s were harvested and washed twice with cold PBS followed by staining with 5 μL V-FITC and 5 μL PI for 10 min. The apoptosis state in each group was detected by FCM and the data were analyzed with FlowJo software. This experiment is repeated three times.

2.9. Detection of mitochondrial membrane potential ($\Delta\psi_m$) and reactive oxygen species (ROS)

Mitochondrial membrane potential detection was performed as previously reported and examined by FCM after Rh123 staining [13]. Adherent cells were treated with indicated dose of PoPx(0, 12.5 25, 50, 100, 200 μg/mL) for 48 h. The medium was

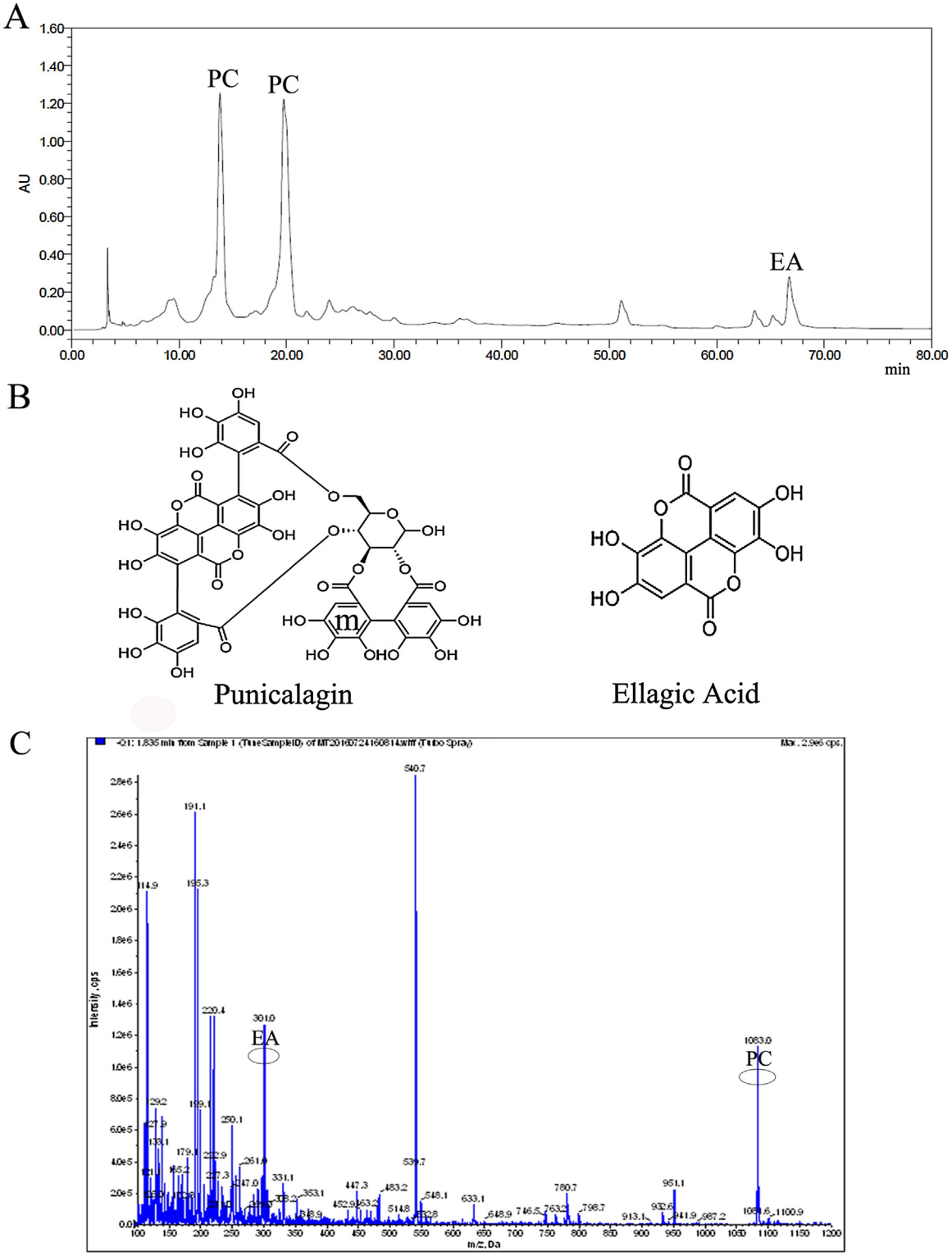

Fig. 1. HPLC and MS of PoPx. (A) HPLC analysis of PC (peak 1 and peak 2) and EA (peak 3) in PoPx. (B)The molecular structure of PC and EA. (C) The molecular weight of EA (ellagic acid $[M-H]^-$:301.0) and PC(punicalagin $[M-H]^-$:1083.0).

subsequently removed to collect cells by digestion and centrifugation. Then the cells were stained with 10 μM Rh123 and incubated at 37 °C in the dark for 30 min. The fluorescence was detected by FCM and the changes of mitochondrial membrane potential ($\Delta\psi_m$) were analyzed.

Similarly, the intracellular ROS level was assessed by DCFH-DA probe. After treatment with PoPx for 48 h, the cells were incubated with 10 μM DCFH-DA at 37 °C in the dark for 30 min. Then the stained cells were harvested and washed twice with cold PBS, and the fluorescence was subsequently detected by FCM. This experiment is repeated three times.

2.10. Wound-healing migration assay

Migration and invasion are two important steps in cancer metastasis. To investigate the potential of PoPx to inhibit prostate cancer metastasis, we did wound-healing assay to study PoPx's effects on cancer cells migration as described previously [14]. Briefly, when grow to approximately 80% confluence in monolayer, TRAMP-C1 cells were scraped by a sterile 10 μL pipette tip and the cell debris was washed with PBS. Fresh medium containing 2% FBS and various concentrations of PoPx were then added and incubated for 48 h. Cells migrated into the wounded area were assessed by photographing under a microscope (Zeiss, Germany). The inhibition of migration by PoPx was quantified. The assay was replicated 3 times.

2.11. Boyden chamber migration and invasion assay

A Boyden chamber (8 μm pore size) migration assay was conducted as previously described, with a few modifications [14]. In brief, 100 μL serum-free medium containing 1.0×10^5 cells were added into the top chamber of 24-well transwell (Millipore), and 600 μL medium containing 10% FBS was added to the bottom chamber. Various concentrations of PoPx (0, 50, 100, 200 μg/mL) were added into both chambers. The cells were allowed to migrate for approximately 48 h and non-migrated cells in the top chamber were removed by cotton swab. Then the migrated cells attached to the lower surface of the transwell membrane were fixed in methanol and stained with 0.5% crystal violet for 15 min. Migrated cells in 3 fields selected randomly were counted and photographed under a light microscope.

Invasion assay was performed according to previous studies [14]. Briefly, Matrigel (BD Biosciences, U.S.A.) was diluted 1:3 in PBS and then added to the upper surface (60 μL per well) of 24-well transwell plate and incubated at 37 °C for polymerization. Then he lower compartment of the chamber was filled with 600 μL medium containing 10% FBS. 1.0×10^5 cells in 100 μL serum-free medium were placed in the upper part of each transwell and treated with designated concentrations of PoPx (0, 50, 100, 200 μg/mL). After 48 h incubation, non-invaded cells on the upper side were removed by cotton swab and those invaded to the bottom side of the transwell were fixed with methanol and stained with 0.5% crystal violet for 15 min. Invaded cells were counted under a light microscope and the images were taken. Invaded cells in five independent areas per well were counted and inhibition rate of invasion was then calculated. The two assays were replicated 3 times.

2.12. Western blot analysis

To determine the effect of PoPx on the signaling pathway involved in this study, some proteins in TRAMP-C1 cells were evaluated by western blot as described previously [15]. Firstly, TRAMP-C1 cells were treated with designated concentrations (0, 50,100, 200 μg/mL) of PoPx for 48 h. Then the cells were washed twice with cold PBS and lysed in RIPA containing cocktail for 30 min on ice. After centrifugation at 13300 rpm at 4 °C for 15 min, the supernatant of the lysate was harvested. Protein concentration was determined by BCA protein Assay Kit (Pierce), using known amounts of bovine serum albumin (BSA) to standardize protein concentration and equalized before loading. Cell lysates containing equal amounts of protein (20–40 μg) from each sample were subjected to sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience, Piscataway, N.J.). Membranes were blocked for 1 h at room temperature with 5% skimmed milk (in PBST) and then incubated overnight at 4 °C with each primary antibody. After three washes with PBST (5 min each), membrane was incubation with the relevant HRP-conjugated secondary antibodies for 1 h at room temperature followed by three 5-min washes with PBST. The signal of the bands was detected by using an enhanced chemiluminescence(ECL) kit (Amersham Bioscience, Piscataway, N.J.) per the manufacturer's instructions. The expression of protein was quantified by Image-Pro-Plus 6.0 software.

2.13. Statistical analysis

Cell culture-based experiments were done in biological triplicates and quantifications of staining were done on sections in at least three different views. Data were expressed as means ± SD and the analysis was compared by one-way analysis of variance (ANOVA) followed by Dunnett-*t* using SPSS 16.0 software. Statistically significant p values were considered at: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. Analysis of punicalagin and ellagic acid in PoPx by HPLC and MS

PoPx was comprehensively analyzed by the combination of HPLC and MS. The prepared PoPx sample was firstly analyzed by using the optimized HPLC method and the component were matched by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012A). As shown in Fig. 1A, the main constituents of PoPx were determined as punicalagin (PC, peak 1 and peak 2) and ellagic acid (EA, peak 3) by comparing their retention time with standard substance. The chemical structures of PC and EA were shown in Fig. 1B. And the molecular weight of EA (ellagic acid [M−H]: 301.0) and PC (punicalagin [M−H]: 1083.0) were detected by mass spectrum (Fig. 1C).

The content of PC and EA in PoPx were computed based on the regression equation and the relevant AUC (area under the curve) of each component. Linear regression model was fit for the analysis of PC and EA, both of which were expressed by plotting AUC (y) of the peak against the concentrations (x, mg/mL) (Table 1). Both compound showed high linearity during investigated ranges. Based on the linear regression, the contents of PC and EA were 479.82 mg/g and 7.53 mg/g in PoPx, respectively.

Table 1
Linear range, regression equation and R^2 of EA and PC.

Component	Linear range (mg/mL)	Regression equation	*R2*
EA	50–500	$y^a = 149530x + 2 \times 10^6$	0.9935
PC	125–2000	$y^a = 2 \times 10^7 x - 6 \times 10^6$	0.971

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

3.2. The effects of PoPx on proliferation and the morphology of prostate cancer cells

To evaluate the anti-proliferative effect of PoPx, human prostate cancer cell lines DU145, PC3 and mouse prostate cancer cell TRAMP-C1 were treated with designated concentrations of PoPx (0, 12.5, 25, 50, 100 and 200 μg/mL) for 24 h, 48 h and 72 h, respectively. As revealed in Fig. 2A, PoPx showed good inhibition on TRAMP-C1 cell proliferation in a time and concentration dependent manner. Although the growth of DU145 and PC3 was not inhibited when exposed to low concentrations of PoPx for 24 h, the higher concentration and longer time treatment showed good inhibition of cell growth. According to Yujue Li [16], the pomegranate peel extract (PoPx) has a relative high selectivity index and its IC_{50} values on normal cells was more than 400 μg/mL, which indicated that PoPx might have high safety profiles.

To further investigate the long-term effects of PoPx on cell survival, we conducted clonogenic assay. As shown in Fig. 1B, long-time PoPx exposure led to reduced clone formation of DU145, PC3 and TRAMP-C1 cell, and there is no visible colony in the high dose

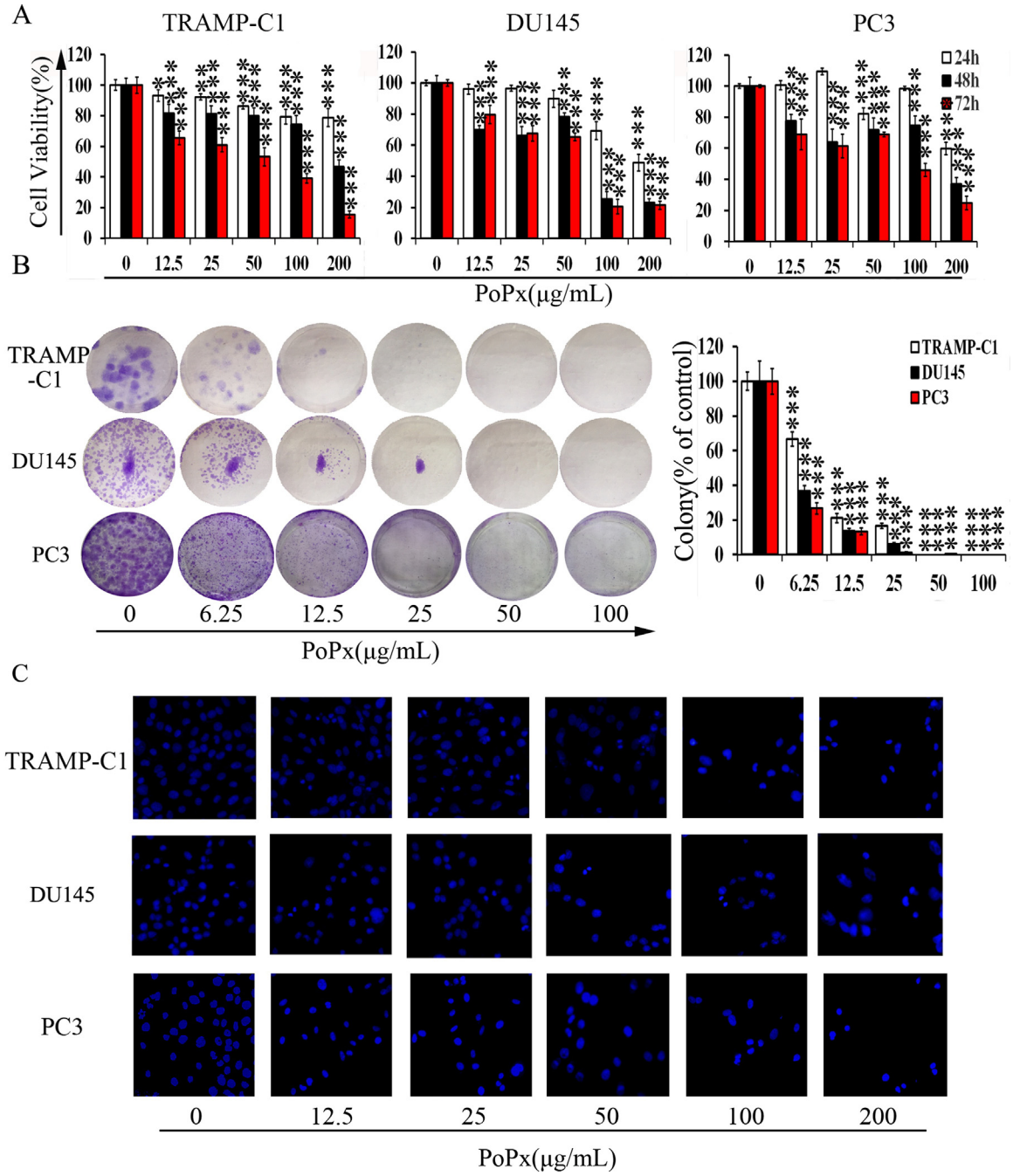

Fig. 2. The effects of PoPx on PCa cells proliferation. (A) TRAMP-C1, DU145 and PC3 cells were incubated with designated concentrations (0–200 μg/mL) of PoPx for 24, 48 and 72 h. Cell viability was determined by MTT assay. Data are expressed as means ± SD from three independent experiments (*$p < 0.05$; **$p < 0.01$;***$p < 0.001$, compared to control). (B) The effects of PoPx (0–100 μg/mL) on colony formation in TRAMP-C1, DU145 and PC3 cells for about 7-10 days, the statistic results of colony formation assays presented as surviving colonies. Data are expressed as means ± SD from three independent experiments (***$p < 0.001$, compared to control). (C) The fluorescence microscopic appearance of Hoechst 33258 staining nuclei of TRAMP-C1 and PC3 cells with various concentrations of PoPx for 48 h (20×). Data are the representative from three experiments.

treatment group. Moreover, the size of the colonies is significantly smaller after PoPx treatment. Thus, the above data implied that PoPx displayed strong cytostatic effects on TRAMP-C1, DU145 and PC3 cells.

3.3. PoPx induced apoptosis of prostate cancer cells

Next, we investigated whether apoptosis contribute to PoPx-induced growth inhibition. As shown in Fig. 2C, Hoechst 33258 staining showed condensed nuclei and nuclear fragmentation in PC3, DU145 and TRAMP-C1 cells after exposure to PoPx for 48 h. To quantify the apoptosis induced by PoPx in TRAMP-C1 cells, we analyzed the cells by FCM after Annexin V-FITC/PI dual-labeling. As revealed in Fig. 3A and B, the apoptosis rate of the cell significantly increased after 48 h PoPx-treatment from 6.03% in vehicle control group to 67.55% in 200 μg/mL treatment group. Bax and Bcl2 are important proteins in the mitochondrial apoptotic pathway. To further confirm the preliminary mechanism of PoPx-induced apoptosis, the expression levels of some apoptosis-related proteins were detected in TRAMP-C1 by western blot. The results in Fig. 3C indicated decreased expression of anti-apoptotic Bcl2 and increased expression of Cleaved caspase 3 and pro-apoptotic Bax after PoPx treatment. Notably, the expression ratio of Bax/Bcl2 increased from 0.53 in vehicle control group to 1.79 in 200 μg/mL treatment group (Fig. 3D). These data indicate that PoPx is able to induce apoptosis of TRAMP-C1 cells, and it might be *via* the mitochondrial apoptotic pathway.

3.4. PoPx treatment decreased mitochondrial transmembrane potential ($\Delta\psi_m$) and ROS production

To further investigate the mechanism of PoPx induced apoptosis in TRAMP-C1 cells, we next detected the changes of $\boldsymbol{\Delta\psi_m}$ by FCM after the fluorescent dye Rh123 staining. The results in Fig. 4A showed that there is a remarkable loss of $\Delta\psi_m$ in TRAMP-C1 after PoPx-treatment for 48 h. Compared with 29.57% in the vehicle control group, the loss of $\boldsymbol{\Delta\psi_m}$ increased to 66.13%, 68.03% and 76.24% after 50, 100 and 200 μg/mL PoPx treatment, respectively.

ROS could regulate numerous vital activities in cell, including transcription factor activation, gene expression, differentiation and cell proliferation, *etc.* [17]. Generation of ROS in cancer cells could stimulate cell death because cancer cells are under much oxidative stress. After PoPx treatment for 48 h, ROS level in TRAMP-C1 was detected by FCM *via* DCFH-DA staining. As displayed in Fig. 4B, ROS level in TRAMP-C1 cells was upregulated a lot after PoPx treatement. Thus, those results revealed that PoPx-induced apoptosis in TRAMP-C1 cells might be *via* mitochondria-mediated apoptotic pathway.

3.5. Effects of PoPx on migration and invasion of TRAMP-C1 cells

Metastasis is the main cause of cancer related death. Migration and invasion are two important steps in cancer metastasis. To investigate the potential effects of PoPx on prostate cancer metastasis, we performed wound-healing and transwell assay on TRAMP-C1 cells. It is obviously to see that the migration

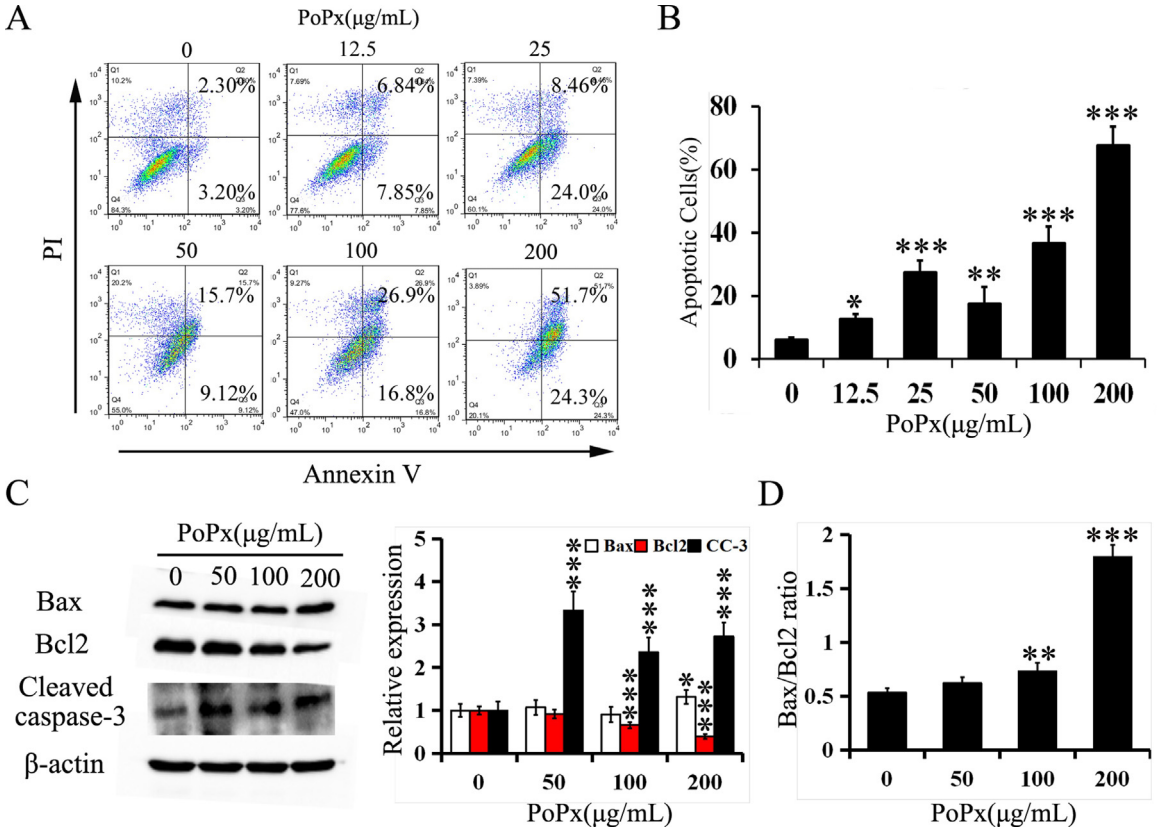

Fig. 3. Induction of apoptosis in TRAMP-C1 cells by PoPx-treatment. (A) TRAMP-C1cells were exposed to PoPx at designated doses for 48 h. (B) The apoptosis rate was treated statistically. Data are expressed as mean ± SD. (*$p < 0.05$; **$p < 0.01$;***$p < 0.001$, compared to control). (C) Western blot analyses of TRAMP-C1cells treated with various concentrations(0, 50, 100 and 200 μg/mL) of PoPx for 48 h to evaluate protein expression of Bcl-2, Bax, Cleaved caspase-3 and β-actin served as a loading control. Protein expression of Bcl-2, Bax and Cleaved caspase-3 were quantified by the densitometry analysis using Image-Pro Plus and normalized against β-actin expression. Statistics of the relative expression of Bax, Bcl-2 and Cleaved caspase-3 after treatment with PoPx are shown. Data are expressed as mean ± SD from three experiments. (***$p < 0.001$, compared to control) (D) The percentage of Bax/Bcl-2 ratio was presented in the bar graphs on the right.

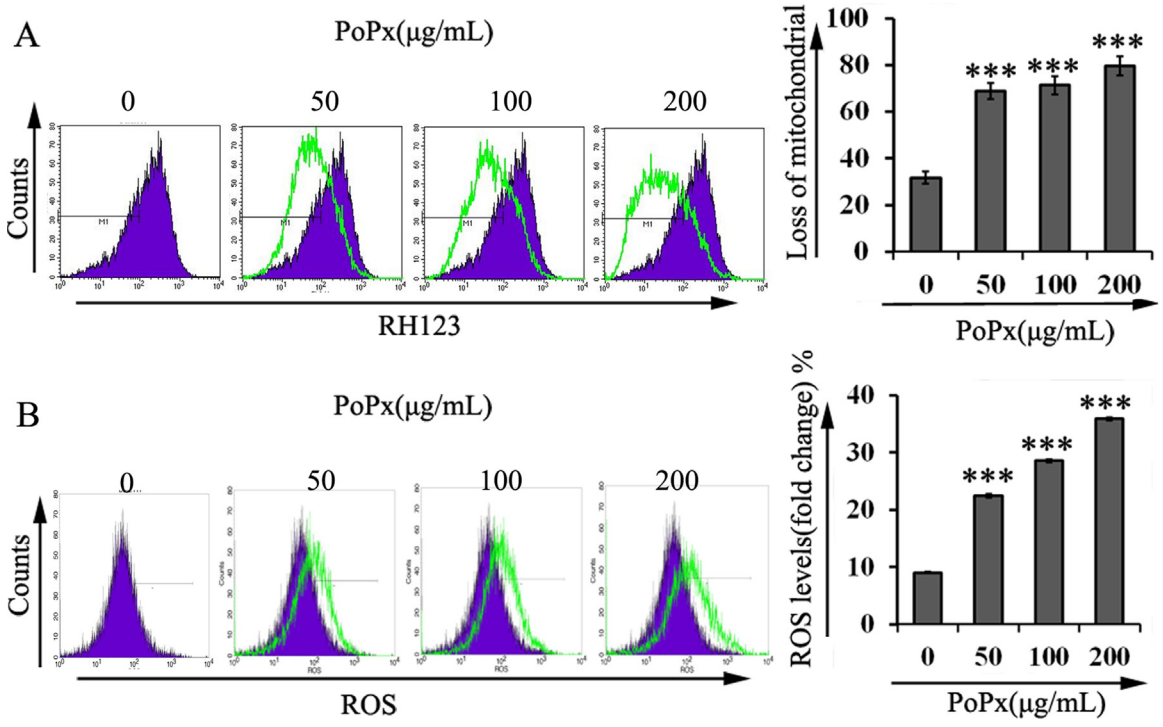

Fig. 4. The changes in the mitochondrial membrane potential (ΔY_m) and the accumulation of ROS. (A) TRAMP-C1 cells were treated with various concentrations of PoPx (0, 50, 100 and 200 μg/mL) for 48 h. The shape filled with purple color was stand for control. (B) The mean fluorescence intensity detected in the mitochondrial membrane potential assay was treated statistically. Data are presented as mean ± SD. (***$p < 0.001$ compared to control) (C)TRAMP-C1 cells were treated with PoPx at indicated doses(0, 50, 100 and 200 μg/mL) for 48 h, followed by analysis of ROS by flow cytometry. The control group was presented as the shape filled with purple color. (D) The variation of ROS was calculated. Data are expressed as mean ± SD. (***$p < 0.001$ compared to control).

capability was suppressed by PoPx (Fig. 5A). A similar result from transwell migration assay also showed that PoPx reduced TRAMP-C1 cells' migration ability (Fig. 5B). Furthermore, results from matrigel invasion assay indicated a significant decrease in the invasion capabilities of TRAMP-C1 cell after PoPx treatment for 48 h, as shown in Fig. 5C.

Tumor metastasis is a complex multi-step process and many proteins are involved in it. To preliminary study the molecular mechanism of PoPx's inhibition on prostate cancer cells migration and invasion, we detected the expression level of several key proteins by western blot. As Fig. 5D indicated, after treatment with PoPx, the expression level of MMP2 and MMP9 in TRAMP-C1 was suppressed significantly compared to vehicle control group. By contrast, expression of TIMP2 was upregulated after PoPx treatment. Altogether, our results implied that PoPx possessed a moderate inhibition on TRAMP-C1 cell migration and invasion.

4. Discussion

Prostate cancer is a big threat to male and lacks effective treatment option. And a lot of pharmaceutical companies and institutes are trying to find novel drugs to treat this devastating disease. Numerous research reports that natural compounds from plants can trigger apoptosis in a lot of cancer cells with different histologic origin [18]. This inspires us to investigate the pharmacological activities of extracts from pomegranate peels on prostate cancer.

Dysregulation of apoptosis is a hallmark of cancer and many anticancer drugs exhibit the activities by inducing cancer cells apoptosis, making it a promising anticancer target [6,7]. FDA has approved Venclexta to treat for chronic lymphocytic leukemia patients. It is a Bcl2 inhibitor and mainly target to the apoptosis pathway [19,20]. Metastasis is the main reason of cancer related death. Thus, we should pay more attention the ability to suppressing metastasis when developing novel anticancer drugs. In this study, we assessed the effects of extract from natural compounds on apoptosis and metastasis. We explored the anticancer effects of PoPx on some prostate cancer cells *in vitro* and the underlying possible mechanisms. We find that PoPx could decrease prostate cell viabilities, induce apoptosis and inhibit migration and invasion.

Firstly, we used MTT assay to assess the activities of PoPx on the growth and proliferation of several prostate cancer cell lines. We find that PoPx inhibited DU145, PC3 and TRAMP-C1 prostate cancer cells viabilities in a dose- and time-dependent manner. To get into the mechanism of PoPx's anti-proliferation effects, we studied PoPx's effects on the apoptosis pathway. Flow cytometry analysis indicated that apoptosis contributed to the decreased cell viability after PoPx treatment. Further studies implied that PoPx might induce apoptosis *via* mitochondrial intrinsic pathway.

There is growing evidence that mitochondrial dysfunction contribute to apoptosis, which is associated with a decreasing mitochondrial transmembrane potential, Δψm [21]. Besides, it's reported that the accumulation of ROS can change the intracellular environment and disrupt the intracellular homeostasis [22]. Disruption of mitochondrial membrane permeability and loss of mitochondrial transmembrane potential ($\boldsymbol{\Delta\psi_m}$) are important events in the intrinsic apoptotic pathway. In this study, we found a pronounced loss of $\boldsymbol{\Delta\psi_m}$ in prostate cancer cells after PoPx treatment. Reactive oxygen species (ROS) are mainly produced in mitochondria and generation of ROS in cancer cells could lead to cell apoptosis because cancer cells are under much oxidative stress. After PoPx treatment, ROS levels in prostate cancer cells increased, which might contribute the apoptosis [23].

To gain more insight into the precise mechanism of PoPx to induce apoptosis, expression level of Bax, Bcl2 and cleaved-caspase-3 were detected, which are crucial signaling regulators of intrinsic apoptosis [24]. Caspase-3 is generally considered as an

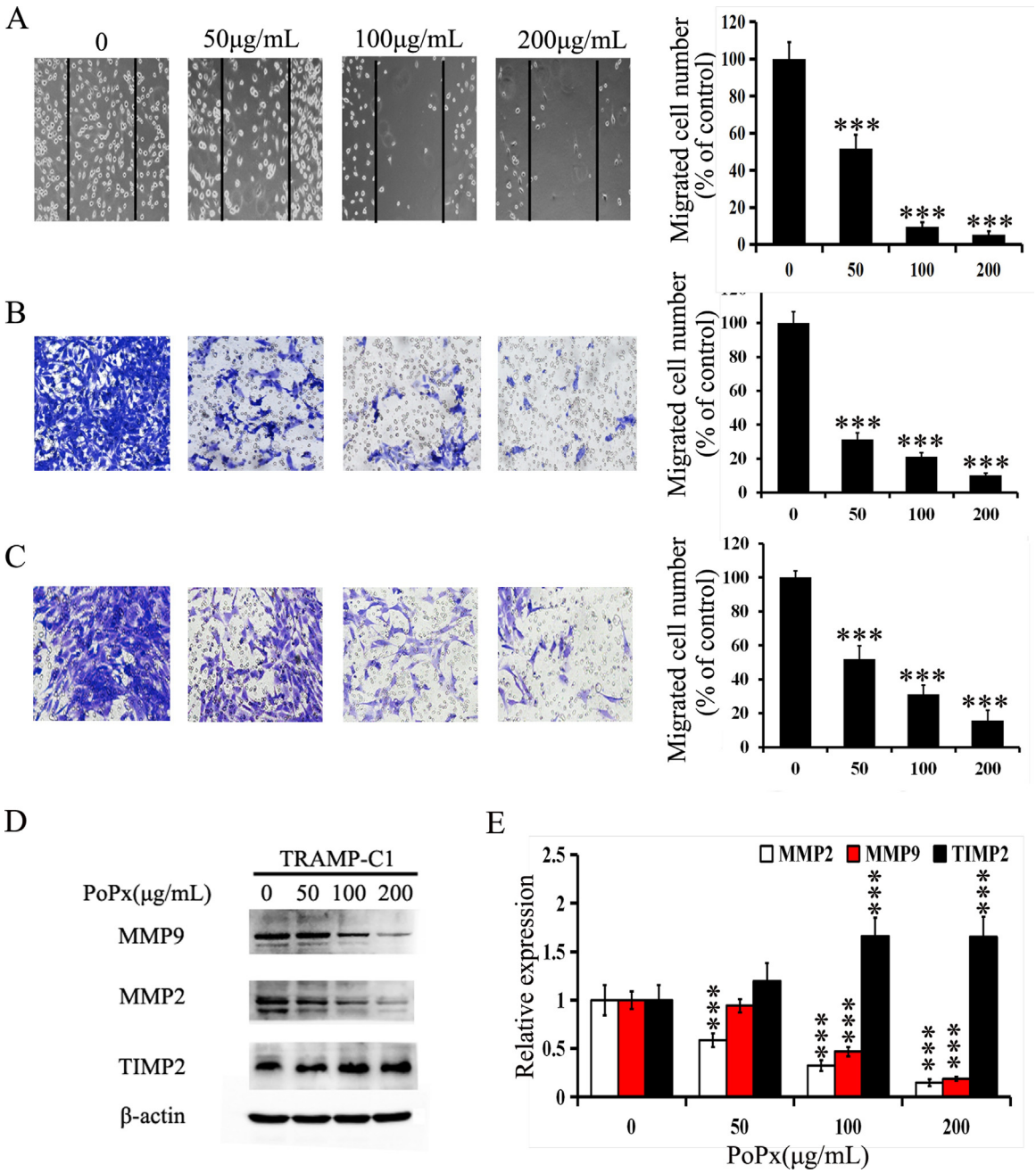

Fig. 5. Inhibition of migratory and invasive in TRAMP-C1 cells by PoPx −treated. (A) TRAMP-C1 cells were seeded on six-well plates. A single scratch was made after the cells grew about 80% confluence. After treatment of PoPx for 48 h, the cells were fixed and photographed (10×). The lines indicate the area occupied by the initial scraping and migrated cells were quantified. (B) TRAMP-C1 cells were seeded in the top chamber of transwell with serum-free medium and treated with vehicle or different concentrations of PoPx. After about 48 h, migrated cells were fixed, stained, photographed and quantified (20×). (C)TRAMP-C1 cells were treated with different concentrations of PoPx and allowed to invade through Matrigel for 48 h. Invaded cell number was counted (20×). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control) (D) TRAMP-C1 cells were treated with different doses of PoPx for 48 h, cells were harvested and western blot assay was carried out to detect the expression of MMP2, MMP9 and TIMP2. β-actin served as loading control. (E) Protein expression of MMP-2, MMP-9 and TIMP2 were quantified by the densitometry analysis using Image-Pro Plus and normalized against β-actin expression. Data are expressed as mean ± SD from three experiments. (*$p < 0.01$, ***$p < 0.001$, compared to control).

important effector protease that is cleaved and activated during apoptosis [25]. Bcl2 family protein plays important role in mitochondrial-dependent apoptosis and alteration in the balance between anti-apoptotic Bcl2 and pro-apoptotic Bax plays the decisive role in cell's susceptibility to apoptosis [26,27]. The data in this study showed that PoPx-induced apoptosis was accompanied by the activation of caspase-3, increased Bax/Bcl2 ratio. The present work indicated that PoPx-induced apoptosis might be *via* mitochondria-dependent intrinsic pathway. Notably, these observations are in accordance with apoptosis of other cancer cells induced by pomegranate peel extract reported previously [16,21,28]. These findings suggested that PoPx is able to inhibit the growth of TRAMP-C1 by inducing mitochondria-dependent intrinsic apoptosis.

It's known that decreasing cancer cell invasion and migration is one possible strategy to prevent or treat cancer metastasis. Matrix metalloproteinases (MMPs) are critical proteolytic enzymes to destruct the integraty of extracellular matrix in the microenvironment, an important process for successful cancer metastasis [29,30]. Among them, MMP2 and MMP9 are generally secreted into

the extracellular space, and their expressions have been strongly correlated with the invasion of many types of cancer cells and poor patient prognosis [31–34]. Migration and invasion are two important step in metastasis. We carried out some assays to investigate the anti-metastasis ability of PoPx *in vitro*. In both wound healing assay and transwell migration and invasion assay, it's clear that treatment with PoPx significantly restrained TRAMP-C1 cells migration and invasion ability. PoPx treatment also down-regulated MMP2 and MMP9 expression in TRAMP-C1 cells. The present *in vitro* data implied that PoPx might has the potential for controlling cancer metastasis, which need to be further assessed. The balance between MMPs and TIMPs is important for tumor cell invasion and metastasis. Tissue inhibitor of matrix metalloproteinase 2(TIMP2), a specific inhibitor of MMP2, is closely associated with the activation status of MMP2. MMP2/TIMP2 system is believed to play a significant role in tumorigenesis and progression [35] In this study, exposure to PoPx lead to upregulation of TIMP2, which might contribute to the downregulation of MMP2 and MMP9 and decreased prostate cancer migration and invasion.

In summary, we have demonstrated that PoPx presented obvious inhibition effects on the growth and viabilities of prostate cancer cells. PoPx could also induce prostate cancer cells apoptosis, which is accompanied by increased expression of cleaved caspase-3. PoPx treatment also increased the Bax/Bcl2 expression ratio and ROS production level and a loss of $\Delta\psi m$, indicating the involving of mitochondrial-mediated intrinsic apoptotic pathway. Additionally, PoPx can impaired prostate cancer cell migration and invasion, accompanied with a decreased expression of MMP2 and MMP9 and increasing expression of TIMP2, implying the potential of PoPx to treat or prevent cancer metastasis. These findings provide the theoretical basis for the further investigation of PoPx and other natural compound, giving us a better way to find drugs to treat cancer and other disease.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, CA: Cancer J. Clin. 66 (1) (2016) 7–30.
[2] M. Saleem, A novel biomarker for staging human prostate adenocarcinoma: overexpression of matriptase with concomitant loss of its inhibitor, hepatocyte growth factor activator inhibitor-1, Cancer Epidemiol. Biomarkers Prev. 15 (2) (2006) 217–227.
[3] Zhou Long, LiuBin Xia, BaiJun Chen, Qian Liu, ShengQuan Huang, Jiang Zhao, XiaoYan Hu, Bo Song, ZhenXing Yang, LongKun Li, X. Dong, The reverse-mode NCX1 activity inhibitor KB-R7943 promotes prostate cancer cell death by activating the JNK pathway and blocking autophagic flux, Oncotarget 7 (27) (2016) 42059–42070.
[4] Y. Li, C. Guo, J. Yang, J. Wei, J. Xu, S. Cheng, Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract, Food Chem. 96 (2) (2006) 254–260.
[5] G.P. Joshi, M.S. Rawat, V.K. Bisht, J.S. Negi, P. Singh, Chemical constituents of asparagus, Pharmacogn. Rev. 4 (8) (2010) 215.
[6] D. Hanahan, Robert A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (5) (2011) 646–674.
[7] D.W. Nicholson, From bench to clinic with apoptosis-based therapeutic agents, Nature 407 (2000) 810–816.
[8] G.-Z. Ma, C.-M. Wang, L. Li, N. Ding, X.-L. Gao, Effect of pomegranate peel polyphenols on human prostate cancer PC-3 cells in vivo, Food Sci. Biotechnol. 24 (5) (2015) 1887–1892.
[9] Maria Ferrantini, F. Belardelli, Gene therapy of cancer with interferon: lessons from tumor models and perspectives for clinical applications, Semin. Cancer Biol. 10 (2) (2000) 145–157.
[10] R. Alicia Folgueras, M. Alberto Pendás, M. Luis Sánchez, C. López-otín, Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies, Int. J. Dev. Biol. 48 (2004) 411–424.
[11] F. Yang, M. Hu, Q. Lei, Y. Xia, Y. Zhu, X. Song, Y. Li, H. Jie, C. Liu, Y. Xiong, Z. Zuo, A. Zeng, Y. Li, L. Yu, G. Shen, D. Wang, Y. Xie, T. Ye, Y. Wei, Nifuroxazide induces apoptosis and impairs pulmonary metastasis in breast cancer model, Cell Death Dis. 6 (2015) e1701.
[12] Y. Li, F. Yang, W. Zheng, M. Hu, J. Wang, S. Ma, Y. Deng, Y. Luo, T. Ye, W. Yin, Punica granatum (pomegranate) leaves extract induces apoptosis through mitochondrial intrinsic pathway and inhibits migration and invasion in non-small cell lung cancer in vitro, Biomed. Pharmacother. 80 (2016) 227–235.
[13] J.M. Shi, H.J. Huang, S.X. Qiu, S.X. Feng, X.E. Li, Griffipavixanthone from Garcinia oblongifolia champ induces cell apoptosis in human non-small-cell lung cancer H520 cells in vitro, Molecules 19 (2) (2014) 1422–1431.
[14] R. Riahi, Y. Yang, D.D. Zhang, P.K. Wong, Advances in wound-healing assays for probing collective cell migration, J. Lab. Autom. 17 (1) (2012) 59–65.
[15] S.W. Shin, J.W. Park, Ursolic acid sensitizes prostate cancer cells to TRAIL-mediated apoptosis, Biochim. Biophys. Acta 1833 (3) (2013) 723–730.
[16] Y. Li, T. Ye, F. Yang, M. Hu, L. Liang, H. He, Z. Li, A. Zeng, Y. Li, Y. Yao, Y. Xie, Z. An, S. Li, Punica granatum (pomegranate) peel extract exerts potent antitumor and anti-metastasis activity in thyroid cancer, RSC Adv. 6 (87) (2016) 84523–84535.
[17] Y.H. Han, W.H. Park, Pyrogallol-induced calf pulmonary arterial endothelial cell death via caspase-dependent apoptosis and GSH depletion, Food Chem. Toxicol. 48 (2) (2010) 558–563.
[18] T. Kuno, T. Tsukamoto, A. Hara, T. Tanaka, Cancer chemoprevention through the induction of apoptosis by natural compounds, J. Biophys. Chem. 03 (02) (2012) 156–173.
[19] S. Elmore, Apoptosis a review of programmed cell death, Toxicol. Pathol. 35 (4) (2007) 495–516.
[20] Y.-Q. Guan, Z. Li, J. Chen, H. Tao, W. Wang, Z. Zheng, L. Li, J.-M. Liu, Pathway of programmed cell death in HeLa cells induced by polymeric anti-cancer drugs, Biomaterials 32 (14) (2011) 3637–3646.
[21] J. Li, F. Zhang, S. Wang, A polysaccharide from pomegranate peels induces the apoptosis of human osteosarcoma cells via the mitochondrial apoptotic pathway, Tumor Biol. 35 (8) (2014) 7475–7482.
[22] S. Fulda, A.M. Gorman, O. Hori, A. Samali, Cellular stress responses: cell survival and cell death, Int. J. Cell Biol. 2010 (2010) 1–23.
[23] J.-E. Ricci, C. Muñoz-Pinedo, P. Fitzgerald, B. Bailly-Maitre, G.A. Perkins, N. Yadava, I.E. Scheffler, M.H. Ellisman, D.R. Green, Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain, Cell 117 (6) (2004) 773–786.
[24] H. Zuo, T. Lin, D. Wang, R. Peng, S. Wang, Y. Gao, X. Xu, Y. Li, S. Wang, L. Zhao, L. Wang, H. Zhou, Neural cell apoptosis induced by microwave exposure through mitochondria-dependent caspase-3 pathway, Int. J. Med. Sci. 11 (5) (2014) 426–435.
[25] B. Liu, Z. Jian, Q. Li, K. Li, Z. Wang, L. Liu, L. Tang, X. Yi, H. Wang, C. Li, T. Gao, Baicalein protects Human melanocytes from H2O2-induced apoptosis via inhibiting mitochondria-dependent caspase activation and the p38 MAPK pathway, Free Radic. Biol. Med. 53 (2) (2012) 183–193.
[26] Y. Shi, Mechanisms of caspase activation and inhibition during apoptosis, Mol. Cell 9 (3) (2002) 459–470.
[27] L.D. Walensky, BCL-2 in the crosshairs: tipping the balance of life and death, Cell Death Differ. 13 (8) (2006) 1339–1350.
[28] Bingbing Song, Jia Li, J. Li, Pomegranate peel extract polyphenols induced apoptosis in human hepatoma cells by mitochondrial pathway, Food Chem. Toxicol. 93 (2016) 158–166.
[29] Alicia R. Folgueras, Alberto M. Pendás, Luis M. Sánchez, C. López-Otín, Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies, Matrix Metalloproteinases Cancer 48 (2004) 411–424.
[30] M. Ferrantini, F. Belardelli, Gene therapy of cancer with interferon: lessons from tumor models and perspectives for clinical applications, Semin. Cancer Biol. 10 (2) (2000) 145–157.
[31] Eun-Sook Kim, Jong-Sook Kim, Sang Geon Kim, Sejin Hwang, Chang Ho Lee, A. Moon, Sphingosine 1-phosphate regulates matrix metalloproteinase-9 expression and breast cell invasion through $S1P_3$–Gαq coupling, J. Cell Sci. 124 (2011) 2220–2230.
[32] K.M. Devine, Y. Smicun, J.M. Hope, D.A. Fishman, S1P induced changes in epithelial ovarian cancer proteolysis, invasion, and attachment are mediated by Gi and Rac, Gynecol. Oncol. 110 (2) (2008) 237–245.
[33] Xing-Guang Zhang, L.U. Xiao-Feng, Xiu-Ming Jiao, B.I.N. Chen, J.-X. Wu, PLK1 gene suppresses cell invasion of undifferentiated thyroid carcinoma through the inhibition of CD44v6, MMP-2 and MMP-9, Exp. Ther. Med. 4 (2012) 1005–1009.
[34] C. Gialeli, A.D. Theocharis, N.K. Karamanos, Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting, FEBS J. 278 (1) (2011) 16–27.
[35] P. Srivastava, T.A. Lone, R. Kapoor, R.D. Mittal, Association of promoter polymorphisms in MMP2 and TIMP2 with prostate cancer susceptibility in North India, Arch. Med. Res. 43 (2) (2012) 117–124.