

Autophagy and apoptotic machinery caused by *Polygonum cuspidatum* extract in cisplatin-resistant human oral cancer CAR cells

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Abstract. *Polygonum cuspidatum* (Hu Zhang) is a traditional Chinese medicine (TCM) and has been revealed to exert anti-cancer, anti-angiogenesis, anti-human immunodeficiency virus (HIV), anti-hepatitis B virus, anti-microbial, anti-inflammatory, and neuro-protective bio-activities. However, the effect of *P. cuspidatum* extract (PCE) on drug-resistant human oral cancer cells regarding cell death is not fully understood yet. The present study was undertaken to explore the induction of autophagic and apoptotic cell death and to investigate their underlying molecular mechanisms in PCE-treated cisplatin-resistant human oral cancer CAR cells. Our results revealed that PCE was determined via HPLC analytic method,

and it was revealed that resveratrol may be a major compound in PCE. The data also demonstrated that PCE reduced CAR cell viability in a concentration- and time-dependent response via an MTT assay. PCE had an extremely low toxicity in human normal gingival fibroblasts (HGF). Autophagic and apoptotic cell death was found after PCE treatment by morphological determination. PCE was revealed to induce autophagy as determined using acridine orange (AO), LC3-GFP, monodansylcadaverine (MDC) and LysoTracker Red staining in CAR cells. In addition, PCE was revealed to induce apoptosis in CAR cells via 4',6-diamidino-2-phenylindole (DAPI)/terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) double staining. PCE significantly stimulated caspase-9 and -3 activities as revealed using caspase activity assays. PCE markedly increased the protein levels of Atg5, Atg7, Atg12, Beclin-1, LC3, Bax and cleaved caspase-3, while it decreased the protein expression of Bcl-2 in CAR cells as determined by western blotting. In conclusion, our findings are the first to suggest that PCE may be potentially efficacious for the treatment of cisplatin-resistant human oral cancer.

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Introduction

Apoptosis and autophagy are the major processes of the programmed cell death (PCD) and play important roles in cellular homeostasis and diseases (1,2). Induction of autophagic or apoptotic death in tumor cells is one of the best strategies in chemotherapy (3,4). Autophagy is a well-known double-edged sword, promoting survival and/or inducing cell death (5,6). Autophagy-mediated programmed cell death (autophagic

PCD) is elicited when the cells undergo stress such as cellular damage, nutrient starvation, aging, and pathogen infection (5). Autophagy is characterized by an increase in autophagosomes or autophagic vesicles (double-membrane vesicles), which is mediated by phosphatidylinositol 3-kinase (PI3K) class III, Beclin-1 and autophagy-related protein 14 (Atg14) signals. In addition, autophagosome membrane engagement is complied with the Atg16L1, Atg12, Atg7, Atg5, and LC3 ubiquitin-like conjugation systems. Finally, the autophagosome fuses with the lysosome thus forming an autophagolysosome and degradation of the captured proteins or organelles is carried out by lysosomal enzymes (7). In contrast, apoptosis is characterized by DNA condensation and fragmentation, and the blebbing of nuclear and apoptotic bodies (8,9). The regulators of apoptotic death are Bcl-2 family molecules and caspase cascade (8,10). Pro-apoptotic Bcl-2 family proteins (such as Bax, Bak, Bim and Bid) and anti-apoptotic proteins (such as Bcl-2, Bcl-xL and Mcl-1) can regulate the process of the apoptotic pathway by the ratio of pro-apoptotic Bcl-2 family proteins/anti-apoptotic proteins (10,11).

Polygonum cuspidatum Sieb. et Zucc. (Hu Zhang) is a herbaceous perennial plant found in Taiwan, China, Japan, and America and belongs to the family *Polygonaceae* (12,13). *P. cuspidatum* has been detected to have various phytochemicals, including essential oils, quinones, stilbenes, flavonoids, coumarins and lignans (12-14). *P. cuspidatum* has been used to remove jaundice and clear heat-toxin, to promote blood circulation, dispel stasis, expel wind and dampness, to dissipate phlegm, and to suppress coughing in traditional Chinese medicine (TCM) treatments (12). Its clinical applications also include anti-hepatitis, amenorrhea and leucorrhea therapy, arthralgia therapy, and snake bite therapy (12-14). *In vitro* and *in vivo* pharmacological studies have demonstrated that the extract from *P. cuspidatum* has anti-angiogenesis (15), anti-viral (13,16), anti-microbial (17,18), anti-inflammatory (19-21), and neuro-protective properties (12,22). *P. cuspidatum* extract has exhibited antiproliferative effects against various human cancer cells of HL-60, A549, H1650, L-02, HepG2, SHZ-888 and MCF-7/ADM cells (23-28). The methanol and ethyl acetate extracts of *P. cuspidatum* have been revealed to trigger oral cancer KB cell apoptosis through caspase-3 activation and the regulation of specificity protein 1 (23). Although the various anticancer effects of *P. cuspidatum* have been investigated, its underlying mechanism of autophagy and apoptosis on drug-resistant human oral cancer cells is still unclear. The present study aimed to determine the mechanisms of autophagy and apoptosis induced by the ethanol extract of *P. cuspidatum* (PCE) in cisplatin-resistant human oral cancer CAR cells.

Materials and methods

Plant extraction procedures and analytic method. Dried *Polygonum cuspidatum* Sieb. et Zucc. (Fig. 1A) was obtained from a traditional Chinese medicine drugstore in Taichung, Taiwan. The sample was powdered to a homogeneous size through a 60-mesh filter in a mill and then sieved. The powder (100 g) was extracted twice by 75% ethanol for 60 min under reflux at room temperature. The ethanol extract of *P. cuspidatum* (PCE) was obtained by combining the filtrates

dried in a vacuum at 45°C, and then collecting 40.32 g of brownish viscous residue, as previously described (29). The analysis was carried out via high-performance liquid chromatography (HPLC) analysis, as previously described (30,31), using a Shimadzu LC-20A system consisting of a CBM-20A HPLC pump, a FRC-10A autosampler, UV and PDA detectors, and Merck Purospher STAR RP-18 end-capped 250-4.6 mm (5 μ m) column. Chromatograms were monitored at 280 nm using a UV detector. H₂O (containing 0.2% formic acid) was used as solvent A and acetonitrile was used as solvent B. Following the injection of 10 μ l of the sample, the flow rate of the mobile phases was maintained at 1 ml/min. A linear HPLC gradient was employed: i) 0.0-10.0 min linear gradient from 10 to 40% of solvent B; ii) 10.0-32.0 min linear gradient from 40 to 85% of solvent B; iii) 32.0-40.0 min isocratic at 85% of solvent B. Furthermore, the PCE was re-suspended and dissolved in dimethyl sulfoxide (DMSO) and used for further *in vitro* experiments.

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 1:1 medium, fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin were purchased from HyClone; GE Healthcare Life Sciences (Logan, UT, USA). Cisplatin, DMSO, monodansylcadaverine (MDC), thiazolyl blue tetrazolium bromide (MTT), and resveratrol were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Acridine orange (AO), 4',6-diamidino-2-phenylindole (DAPI), LysoTracker Red DND-99, and trypsin-EDTA were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The primary antibodies [anti-Atg5 (cat. no. GTX113309), anti-Atg7 (cat. no. GTX113613), anti-Atg12 (cat. no. GTX629815), anti-Beclin-1 (cat. no. GTX631396), anti-LC3 (cat. no. GTX39752), anti-Bax (cat. no. GTX109683), anti-Bcl-2 (cat. no. GTX100064), anti-caspase-3 (cat. no. GTX110543) (all 1:1,000 dilution) and anti- β -actin (cat. no. GTX109639) (1:5,000 dilution)] and the anti-rabbit (cat. no. GTX213110-01) or anti-mouse (cat. no. GTX213111-01) IgG-horseradish peroxidase (HRP) secondary antibodies (1:10,000 dilution) were all purchased from GeneTex International Corporation (Hsinchu, Taiwan).

Cell culture. The cisplatin-resistant CAR cells were established as previous methods (3,32-39). The parental human tongue squamous cell carcinoma CAL 27 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) after increasing exposure to 10-80 μ M of cisplatin for 10 cycles and at least stably resistant to 80 μ M cisplatin. CAR cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine and 80 μ M cisplatin. Normal human primary gingival fibroblast (HGF) was purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany) and cultivated in DMEM/F12 1:1 medium with 10% FBS, 100 μ g/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine. All cells were cultured in a 37°C humidified incubator with 5% CO₂.

Cell viability assay and morphological changes. CAR or HGF cells (1 \times 10⁴ cells/well) were plated on 96-well plates and exposed to 50, 100, 150 and 200 μ g/ml of PCE for 24 and 48 h. At the end of treatment, each medium containing 500 μ g/ml MTT

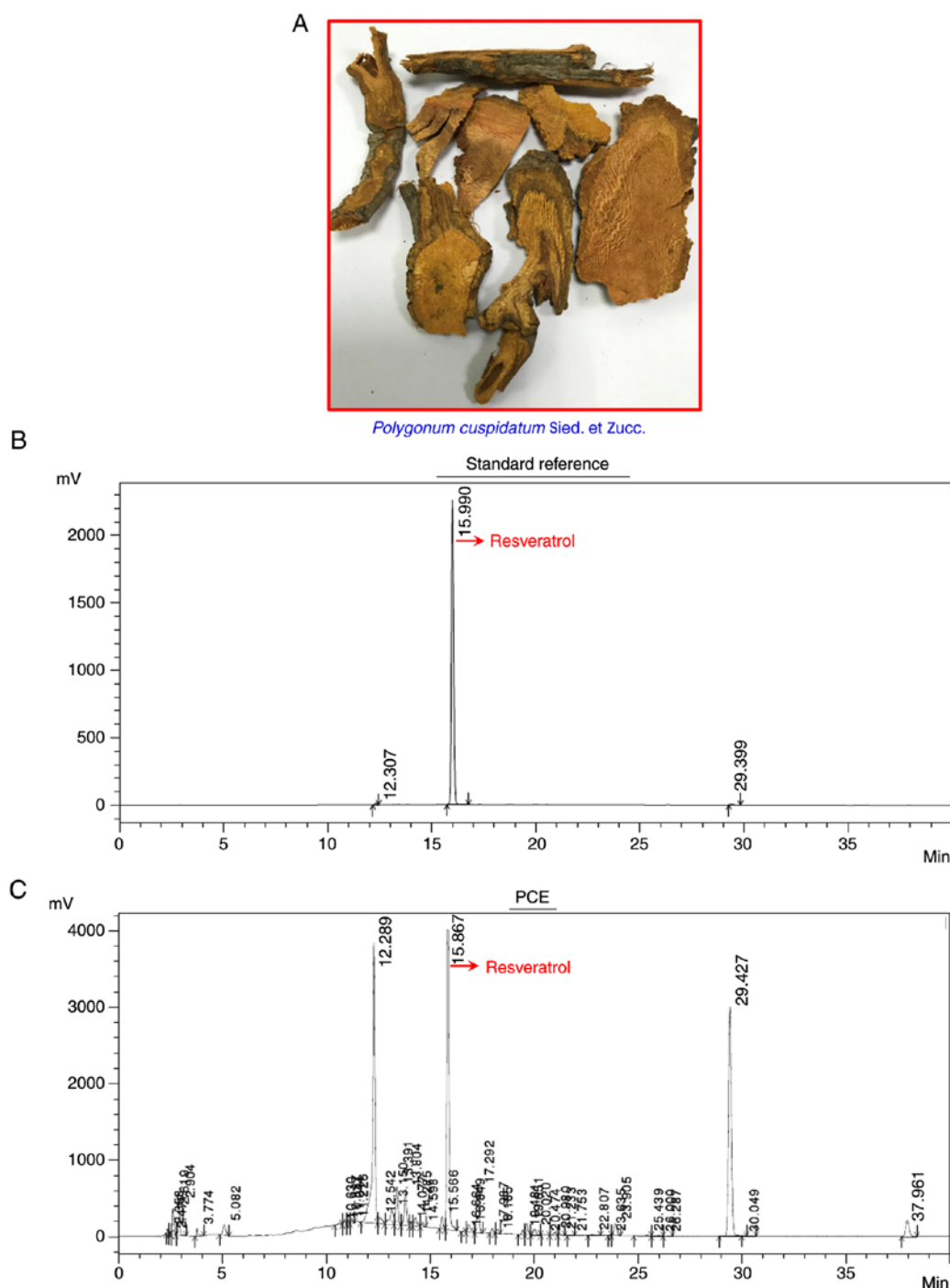


Figure 1. *Polygonum cuspidatum* and HPLC pattern of resveratrol and PCE. (A) Materia medica of the roots of *Polygonum cuspidatum* Sieb. et Zucc. (B) Standard reference: Resveratrol (peak at 15.990 min). (C) The HPLC chromatogram of ethanol extract of PCE revealed a retention time at 15.867 min. HPLC, high performance liquid chromatography; PCE, *P. cuspidatum* extract.

solution was added for an additional 3 h before the medium was discarded from each well. The blue formazan product was dissolved by 100 μ l DMSO, and the optical density was spectrophotometrically detected at an absorbance of 570 nm, as previously described (40,41). Cell morphological changes (autophagic vacuoles and apoptotic characteristics) were visualized and photographed via a phase-contrast microscope, as previously described (40,42).

Autophagy assays. CAR cells (5×10^4 cells/ml) were plated on sterile coverslips in tissue culture plates and then treated with

150 μ g/ml PCE for 24 h. Cells were then individually stained with either 1 μ g/ml AO, 100 μ M MDC, and 1 μ g/ml LysoTracker Red DND-99 for 15 min, as previously described (35,36). The autophagy marker LC3 was detected via the Premo Autophagy Sensor LC3B-GFP (BacMam 2.0) Kit (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The fluorescent images were immediately monitored and photographed by fluorescence microscopy (Nikon Corp., Melville, NY, USA).

Apoptosis assay. CAR cells (2×10^5 cells/well) plated on 12-well plates were incubated with 150 μ g/ml PCE. After

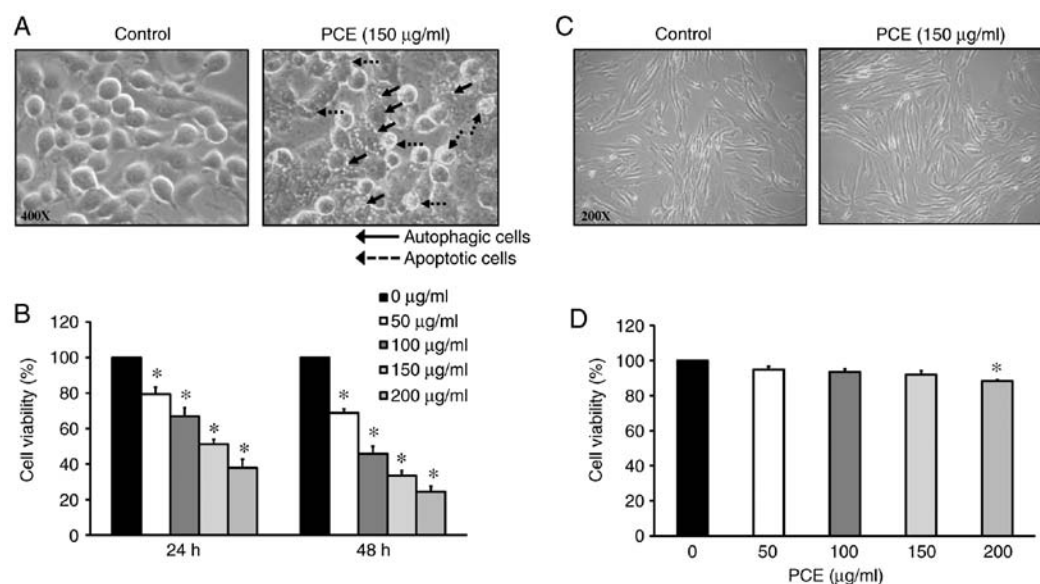


Figure 2. Effect(s) of PCE on CAR and normal HGF cell morphological examination and viability. CAR cells were exposed to the indicated concentrations (0, 50, 100, 150 and 200 $\mu\text{g/ml}$) of PCE for 24 or 48 h. (A) The morphological changes of CAR cells were photographed with a phase-contrast microscope at x400 magnifications. (B) CAR cell viability was assessed by an MTT assay. (C) HGF cells were treated with indicated concentrations of PCE for 24 h. The morphological changes of HGF cells were photographed at x200 magnifications. (D) Cell viability of HGF cells was detected. The results are expressed as the mean \pm SD (n=3). *P<0.05 vs. untreated control. PCE, *P. cuspidatum* extract; HGF, human gingival fibroblasts.

exposure for 24 h, TdT-mediated dUTP-X nick-end labeling (TUNEL) positive cells were determined via the *In Situ* Cell Death Detection kit, Fluorescein (Roche, Sigma-Aldrich; Merck KGaA) according to manufacturer's instructions. The cell image was photographed using a fluorescence microscope after being counterstained with 1 $\mu\text{g/ml}$ DAPI dye, as previously described (3).

Caspase-3 and -9 activity assays. CAR cells (5×10^6 cells/75T flask) were treated with or without 150 $\mu\text{g/ml}$ PCE for 12 and 24 h. Cell lysates were collected, and the supernatant was incubated in the supplied reaction buffer with dithiothreitol and the caspase-specific substrates [Asp-Glu-Val-Asp (DEAD) for caspase-3; Leu-Glu-His-Asp (LEHD) for caspase-9] labeled with *p*-nitroaniline (pNA) at 37°C for 2 h in the dark following the manufacturer's protocols (Caspase-3 and Caspase-9 Colorimetric Assay Kits; R&D Systems, Inc., Minneapolis, MN, USA).

Western blotting. CAR cells (5×10^6 cells/75T flask) were exposed to 0, 150 and 200 $\mu\text{g/ml}$ PCE for 24 h. Whole-cell lysates were isolated with Trident RIPA Lysis Buffer (GeneTex International Corp.), and the protein concentration was quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Equal amounts (40 μg) of protein samples were separated using 10-12% SDS-PAGE, as previously described (42). The separated protein was transferred to an Immobilon-P Transfer Membrane (EMD Millipore, Billerica, MA, USA) via use of electroblotting. The membranes were soaked in 5% skim milk and individually incubated with the primary antibodies, including Atg5, Atg7, Atg12, Beclin-1, LC3, Bax, Bcl-2, caspase-3 and β -actin overnight at 4°C, as well as the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature to hybridize targeted protein using an Immobilon Western Chemiluminescent

HRP Substrate (EMD Millipore). All bands were normalized to the level of β -actin for each lane, and their densitometric quantification was carried out using NIH ImageJ 1.47 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data were reported as the mean \pm standard deviation (SD) of triplicate samples. The significant differences of data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Resveratrol is one of the major compounds in PCE. The result from the HPLC analysis revealed that the major peak of the standard reference (resveratrol) appeared at 15.990 min (Fig. 1B). In addition, the data indicated that PCE had several peaks at various retention time intervals, indicating that PCE possessed multiple components. The retention time of the peak at 15.867 min was identified as resveratrol (Fig. 1C). Therefore, resveratrol may be one of the major compounds in PCE.

PCE inhibits cell viability in cisplatin-resistant human oral cancer CAR cells. PCE promoted the formation of autophagic vacuoles and apoptotic bodies after exposure to 150 $\mu\text{g/ml}$ PCE for 24 h in CAR cells (Fig. 2A). PCE also reduced viable CAR cells in a time- and concentration-dependent manner (Fig. 2B). These findings indicated that autophagy and apoptotic mechanisms may be present in PCE-treated CAR cells. Notably, no morphological changes (Fig. 2C) and cytotoxic effects (Fig. 2D) were found on normal HGF cells. Thus, PCE exerted lower cytotoxicity in normal oral cells and triggered autophagic and apoptotic mechanisms in CAR cells.

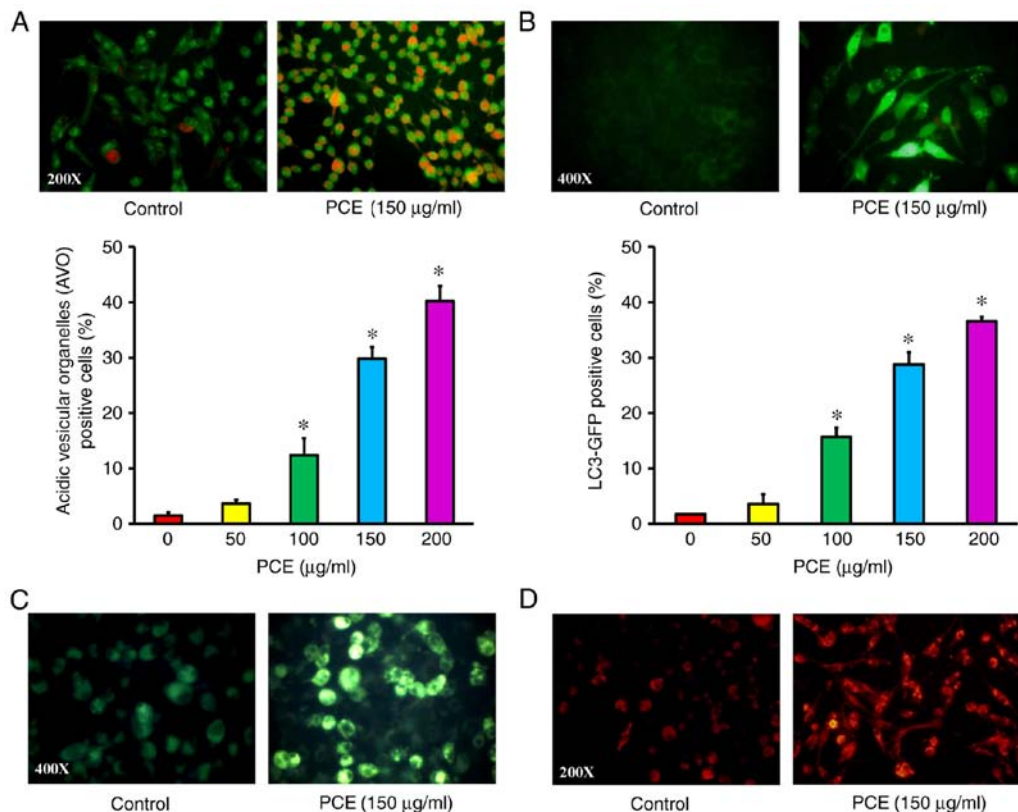


Figure 3. Effect(s) of PCE on autophagy of CAR cells. Cells were exposed to 0, 50, 100, 150 and 200 $\mu\text{g/ml}$ of PCE for 24 h and then were harvested. (A) AO staining was used to detect AVOs (red fluorescence) (at x200 magnifications), and the data was quantified. (B) An LC3B-GFP kit was applied to monitor LC3B expression (green fluorescence) (at x400 magnifications), and the data was quantified. The results are expressed as the mean \pm SD ($n=3$). * $P<0.05$ vs. untreated control. Cells were with or without 150 $\mu\text{g/ml}$ PCE exposure for 24 h. Cells were individually stained with (C) MDC dye (green fluorescence) (at x400 magnifications) and (D) LysoTracker Red DND-99 (red fluorescence) (at x200 magnifications) to detect autophagic vacuoles and lysosomal enzyme activity, respectively, as described in the Materials and methods. PCE, *P. cuspidatum* extract; AO, acridine orange; AVOs, acidic vesicular organelles; MDC, monodansylcadaverine.

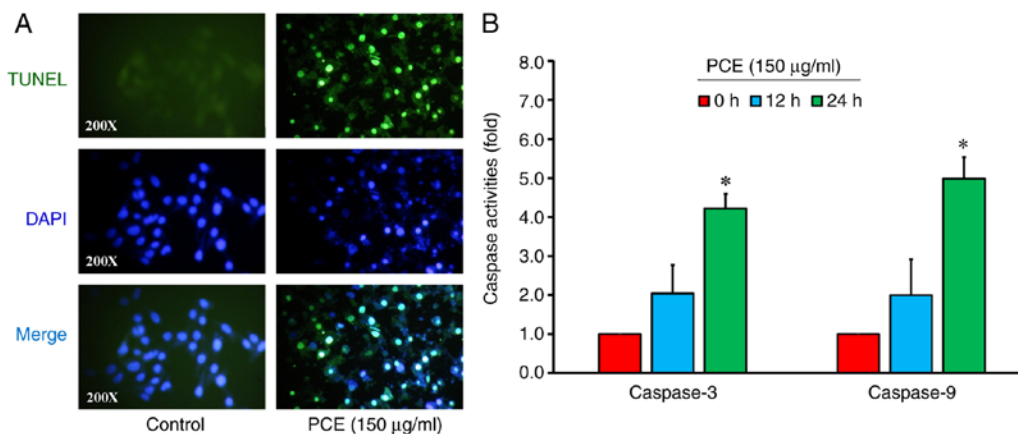


Figure 4. Effect(s) of PCE on apoptotic characteristic(s) of CAR cells. Cells were exposed to 150 $\mu\text{g/ml}$ PCE for 12 or 24 h. (A) Apoptotic DNA breaks and DNA condensation were determined via TUNEL and DAPI staining, at x200 magnifications. (B) The activities of caspase-3 and -9 were estimated, as described in the Materials and methods. The results are expressed as the mean \pm SD ($n=3$). * $P<0.05$ vs. untreated control. PCE, *P. cuspidatum* extract; DAPI, 4',6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

PCE induces autophagy in CAR cells. To determine PCE-induced autophagy, CAR cells were incubated with different concentrations of PCE for 24 h, and then monitored for the formation of acidic vesicular organelles (AVOs) and a punctate pattern of LC3. PCE increased the red fluorescence intensity in the cytoplasm compared to the control cells via AO

staining, indicating that PCE led to AVO occurrence (Fig. 3A). PCE also caused the punctate pattern of LC3-GFP in CAR cells (Fig. 3B). In addition, autophagic evidence was also demonstrated in cells probed with MDC and LysoTracker Red, respectively. Our results revealed that autophagic vacuoles and lysosome activity were individually observed

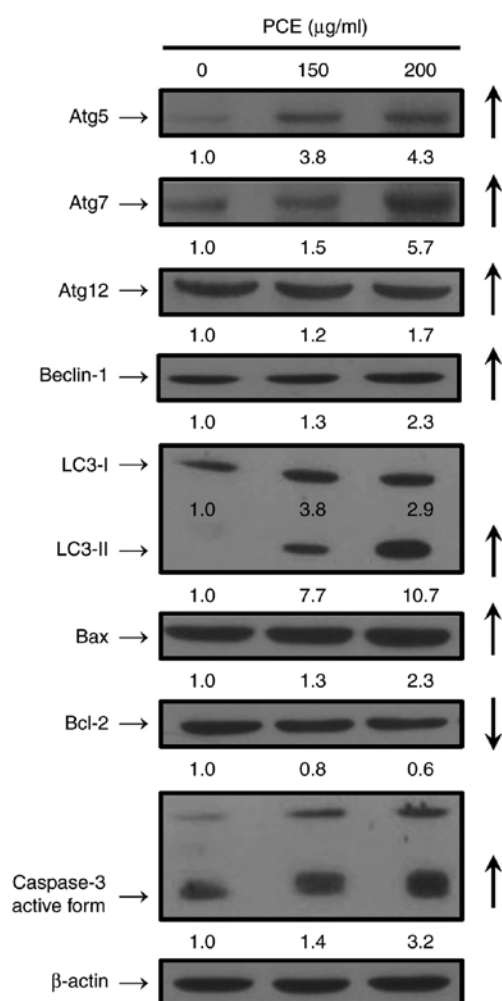


Figure 5. Effect(s) of PCE on autophagy- and apoptosis-regulated protein molecules of CAR cells. The cell lysates from vehicle control or 150 and 200 $\mu\text{g/ml}$ of PCE treatments in CAR cells for 24 h were determined using immunoblot analysis, as described in the Materials and methods. The protein levels of Atg5, Atg7, Atg12, Beclin-1, LC3, Bax, Bcl-2 and caspase-3 were detected via western blotting. β -actin is an internal control. PCE, *P. cuspidatum* extract.

in PCE-treated CAR cells, and the fluorescence intensity of MDC (green fluorescence) (Fig. 3C) and LysoTracker Red (red fluorescence) (Fig. 3D) staining was directly proportional to CAR cells at 150 $\mu\text{g/ml}$ PCE exposure. Therefore, PCE triggered an autophagic response in CAR cells.

PCE induces the apoptotic process in CAR cells. To assess CAR cell apoptosis induced by PCE, the cells were treated with or without 150 $\mu\text{g/ml}$ PCE for 24 h and detected by TUNEL/DAPI staining and caspase-3/-9 activity assays. PCE at 150 $\mu\text{g/ml}$ markedly stimulated apoptotic DNA breaks (TUNEL positive) and elicited the occurrence of DNA fragmentation and condensation in CAR cells (Fig. 4A). To further confirm caspase-3 and caspase-9 signaling in PCE-treated CAR cells, the activity of caspase-3 and caspase-9 was detected. Our data revealed that PCE at 150 $\mu\text{g/ml}$ for 24 h increased the activities of caspase-3 and caspase-9 (Fig. 4B) in CAR cells. The results indicated that PCE-triggered apoptosis resulted from the intrinsic pathway (caspase-3/-9-dependent) in CAR cells.

PCE regulates autophagy- and apoptosis-related protein signaling in CAR cells. PCE at 150 and 200 $\mu\text{g/ml}$ for 24 h increased the protein levels of Atg5, Atg7, Atg12, Beclin-1, and LC3-II in CAR cells (Fig. 5). Based on the key activation of autophagy markers, it is suggested that PCE induced autophagy in CAR cells. Moreover, after treatment with 150 and 200 $\mu\text{g/ml}$ of PCE, the protein levels of Bax and active form of caspase-3 were upregulated, while the level of Bcl-2 was downregulated in CAR cells (Fig. 5). Our findings demonstrated that PCE induced apoptotic death via a mitochondria-dependent pathway in CAR cells.

Discussion

Natural products and traditional Chinese medicine (TCM) have exhibited anticancer activities and low toxicity, and they have been investigated for anticancer activities for some time now (32,43,44). *P. cuspidatum* (Hu Zhang) is a TCM that has been studied for the treatment of various types of cancers, inflammatory diseases, hepatitis virus infection, HIV, and diarrhea (12-14). It has been demonstrated that physcion, emodin, and resveratrol are the main phytochemicals extracted from the *P. cuspidatum* fraction (12-14,20,45). *P. cuspidatum* is a major and abundant resource of resveratrol since the average content of it is $\sim 1\text{-}3\text{ mg/g}$ (13). Our data (Fig. 1B and C) were also consistent with previous studies (13,30). Resveratrol has a wide spectrum of pharmacological activities such as antioxidant, anti-inflammatory, anti-atherosclerotic, and anticancer effects (46-50). Our previous study reported that resveratrol triggered autophagy and apoptosis in CAR cells (3). Herein, the present study is the first to the best of our knowledge, to report that PCE induced autophagy and apoptosis of CAR cells. The cisplatin-resistant subline CAR cells were originally from a human tongue cancer cell line CAL 27 and established using the method of increasing the concentration of cisplatin (to at least 80 μM) according to a previously described method (3,32-37). Furthermore, the differences from the parental CAL 27 cells and CAR cells were investigated. Our findings revealed that CAR cells were resistant to 80 μM cisplatin compared with the parental CAL 27 cells (3,32,34). Notably, multidrug resistance protein 1 (MDR1) revealed a higher expression in CAR cells than in parental CAL 27 cells. Thus far, CAR cells have been a drug-resistant cell platform to test various phytochemicals, traditional Chinese medicine, and novel compounds (3,32-37,39). In the present study, it was firstly demonstrated that PCE reduced cell proliferation in CAR cells (Fig. 2). The half maximal inhibitory concentration (IC_{50}) for 24 and 48 h treatment of PCE in CAR cells were 162.89 ± 6.28 and $110.34 \pm 8.21\text{ }\mu\text{g/ml}$, respectively. PCE exhibited low toxicity to normal HGF cells ($\text{IC}_{50} > 200\text{ }\mu\text{g/ml}$) (Fig. 2). Additionally, our preliminary data indicated that PCE reduced cell viability of the parental CAL 27 cells after 48-h treatment, and the IC_{50} value was $188.39 \pm 4.21\text{ }\mu\text{g/ml}$ (data not shown). As a result, PCE induced more selective cytotoxicity in human cisplatin-resistant oral cancer CAR cells while having a low toxicity to normal cells.

Autophagy and apoptosis are the major routes that lead to cell death (2,5). Our study was undertaken to demonstrate that PCE can induce the formation of autophagic

vesicle and apoptotic bodies (Fig. 2A). It was suggested at the outset that PCE may induce CAR cell death through autophagy and apoptotic pathways. The characteristic of autophagy is to increase autophagosome accumulation in the cytoplasm of cells (5,6,51). The method used for monitoring autophagy in autophagosomes and lysosomal enzyme activity was examining the uptake of fluorescent dyes, such as acridine orange (AO), monodansylcadaverine (MDC), and LysoTracker Red. Furthermore, autophagy-related proteins such as ATGs and LC3 were also detected (4,49,51). In the present study, the results from AO (Fig. 3A), LC3-GFP (Fig. 3B), and MDC staining (Fig. 3C) indicated that PCE induced the formation of autophagic vesicles in a concentration-dependent manner in CAR cells. The LysoTracker Red staining was also used to assess lysosome activity following treatment with PCE (Fig. 3D). PCE also increased the protein level of autophagic proteins, including Atg5, Atg7, Atg12, Beclin-1, and LC3 (Fig. 5) in CAR cells. Notably, 3-methyladenine (3-MA), a specific inhibitor of PI3K kinase class III, inhibited the autophagic vesicle formation induced by PCE (data not shown). Our results support the notion that PCE-induced CAR cell death was mediated through the induction of autophagic death.

In the present study, it was revealed that PCE triggered apoptotic morphological changes in CAR cells (Fig. 2A). PCE induced DNA condensation and fragmentation (Fig. 4A). PCE-induced apoptosis was confirmed by the increase of the activity (Fig. 4B) and protein levels (Fig. 5) of caspase-3 in CAR cells. When the cells undergo apoptotic cell death, the ratio of Bax/Bcl-2 is enhanced leading to cytochrome *c* release from the mitochondria (52). Cytochrome *c* activates the caspase-9/-3 cascade to induce cell apoptosis (10,52). PCE increased the protein level of Bax and decreased the Bcl-2 signal (Fig. 5), indicating that the involvement of the mitochondrial apoptotic pathway contributed to CAR cell death after PCE treatment.

It has been reported that autophagy precedes apoptosis in cervical cancer HeLa cells (53,54) and colorectal DLD1, HT-29 and COLO 201 cells induced by resveratrol (55-57), suggesting that resveratrol triggered apoptotic cell death following the autophagy. Recently, in a separate study, it was demonstrated that resveratrol had an extremely low toxicity in normal HGF cells (3). Resveratrol induced autophagic CAR cell death as observed in AO, LC3-GFP and MDC staining (3). Moreover, resveratrol increased the autophagy-related protein levels (Atg5, Atg7, Atg12, Atg14, Atg16L1, Beclin-1, PI3K class III and LC3), but it decreased rubicon protein expression (3). Resveratrol also induced apoptotic DNA fragmentation, elicited the caspase-3/-9 activities, and increased the protein levels of caspase-3 and -9, and Bax, while it decreased the protein level of Bcl-2 (3,51). The present results are in agreement with previous studies (3,51) in reiterating that resveratrol may be a major autophagic inducer in PCE.

In conclusion, our results demonstrated that PCE treatment of CAR cells induced reduction rather than enhancement of autophagic degradation that led to apoptotic cell death. Thus, the present study provided new insight and motivated us to forge ahead regarding the action(s) of PCE and its molecular mechanism(s). PCE could be used for its potential therapeutic use against drug-resistant oral cancer in the near future.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YLW, CTH, CCL and FAC conceived and designed the experiments; MTH, HCC, YSH, JSY, GKW and JHC performed the experiments. MTH, JSY, JHC, HHC and CCL analyzed the data; YLW, CTH, CCL and FAC wrote and modified the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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