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Original Research

Induction of ROS-dependent apoptotic cell death by platycodin D in human prostate cancer PC3 cells

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Abstract

Background and objective: Platycodin D (PD), a triterpenoid saponin isolated from an edible and medicinal plant *Platycodon grandiflorum*, possesses multiple pharmacological properties. The purpose of this study is to investigate the effect of PD on the growth of PC3 human prostate cancer cells and the underlying molecular mechanisms. **Materials and methods**: Cell viability, apoptosis and mitochondrial membrane potential (MMP) were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, nuclear staining and flow cytometry analysis. To investigate the mechanism of anti-cancer activity of PD, expression of apoptosis regulatory protein, caspase activity, and generation of intracellular reactive oxygen species (ROS) were determined. **Results**: PD treatment reduced PC3 cell proliferation, which was associated with induction of apoptosis, and accompanied by increased expression of Fas, Fas-ligand (FasL) and pro-apoptotic Bax, and decreased expression of anti-apoptotic Bcl-2 and truncation of Bid. PD also inhibited expression of c-FLIP and members of inhibitor of apoptosis protein family, and activated caspases, resulting in an increase in poly (ADP-ribose) polymerase cleavage. However, in the presence of a pan-caspase inhibitor, PD-mediated growth inhibition and apoptosis were significantly protected. PD also destroyed the integrity of mitochondria due to the loss of MMP, leading to cytosolic release of cytochrome *c*. Moreover, the levels of ROS were markedly increased by PD treatment, which was significantly attenuated by the ROS scavenger *N*-acetyl-*L*-cysteine (NAC). Furthermore, NAC fully suppressed PD-induced apoptotic events and cytotoxicity. **Conclusions**: The results of this study show that PD had chemopreventive potential through the induction of ROS-dependent apoptosis in PC3 cells, and that this compound could be useful for developing an effective and selective natural source to inhibit cancer cell proliferation.

Keywords: Platycodin D; Prostate cancer cells; Apoptosis; Caspase; Reactive oxygen species

1. Introduction

Apoptosis is an essential mechanism for maintaining cellular homeostasis, and maintains a healthy balance [1,2]. Cancer occurs as a result of a series of genetic alterations, during which malignant cells will not die and experience abnormal growth [1,3]. Hence, dysregulation of the apoptotic pathway is a prominent hallmark of cancer, which not only promotes carcinogenesis but also makes tumor cells resistant to chemotherapy [4]. Therefore, numerous studies are being conducted focusing on the development of effective apoptosis inducing agents. In particular, it has been reported that numerous naturally occurring agents induce apoptosis in cancer cells without inducing toxicity in normal cells [5,6]. These agents can also replace anti-cancer drugs that have serious side effects because they can regulate specific cell signaling pathways for cancer cell death [2,7].

Platycodin D (PD) is a triterpenoid saponin derived from the root of *Platycodon grandiflorum* (Jacq.) A.DC., which has been traditionally used for the treatment of various ailments diseases [8–11]. Several studies revealed that PD possesses various pharmacological properties due to its antioxidant [12–14], anti-inflammatory [15,16], hepatoprotective [17,18], anti-obesity [19,20], and immunological adjuvant activities [21]. In addition, some studies have described anti-carcinogenic effects of PD against many types of cancers *in vitro* and *in vivo* [22,23]. For example, the concentration of 10~40 μM PD inhibited tumor invasion and metastasis in human oral squamous cell carcinoma, hepatocellular carcinoma, breast carcinoma cells *via* targeting multiple signaling pathways [24–27]. Moreover, PD is known to suppress the proliferation of cancer cells through cell cycle arrest, which was associated with the induction of apoptosis and/or autophagy in several cancer lines [24,28–30]. Furthermore, PD was able to activate reactive oxygen species (ROS)-mediated apoptosis in rat pheochromocytoma, human breast carcinoma and leukemic cells [30–32]. Although the possibility of inducing apoptosis of PD in human prostate cancer cells has been proposed [29], the molecular mechanisms involved in ROS generation have not been reported. Therefore, in the present study, the effect of PD on the proliferation of human prostate cancer PC3 cells and the mechanism of action on the role of ROS were investigated.

2. Materials and methods

2.1 Cell culture and PD treatment

The human prostate cancer PC3 cells, which were obtained from American Type Culture Collection (Manassas, MD, USA), were grown in RPMI 1640 medium (WelGENE Inc., Gyeongsan, Republic of Korea) containing 10% heat-inactivated fetal bovine serum (WelGENE Inc.) at 37 °C in 5% CO_2 humidified incubator. PD was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co.) to prepare a stock solution at a concentration of 20 mM. In order to treat the cells with PD, the stock solution was diluted to appropriate concentrations in the culture medium.

2.2 Cell viability assay

After treatment with various concentration of PD for 48 h, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Invitrogen, Waltham, MA, USA) assay, as described previously [33]. In order to confirm that PD-induced apoptosis was mediated by caspase- and ROS-dependent pathways, cells were pre-treated the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (50 μM z-VAD-fmk; Promega Corporation, Madison, WI, USA) or the ROS scavenger *N*-acetyl-*L*-cysteine (10 mM NAC; Invitrogen) for 1 h, respectively, and then incubated with 20 μM PD for the indicated times. At the same time, changes in cell morphology were observed using a phase contrast microscope (Carl Zeiss, Oberkochen, Germany).

2.3 4'6-Diamidino-2-phenylindole (DAPI) staining

Changes in nuclear morphology were assessed by DAPI staining, a cell-permeable nucleic acid dye. After 48 h of treatment with PD (0–20 μM), the cells were fixed with 3.7% paraformaldehyde (Sigma-Aldrich Chemical Co.), and dyed with DAPI (1 μg/mL, Sigma-Aldrich Chemical Co.) at room temperature for 10 min. Then, the stained cells were observed using a fluorescence microscope (Carl Zeiss).

2.4 Apoptosis analysis using a flow cytometer

After treatment with PD, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V/propidium iodide (PI) (BD Biosciences, San Diego, CA, USA) in the dark for 20 min. Then, the stained cells were analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA), and the populations of annexin V^+/PI^- and annexin V^+/PI^+ cells were considered to represent apoptotic cells, as described previously [34].

2.5 Protein extraction and Western blot analysis

Total proteins were extracted with a protein extraction solution (Intron Biotechnology, Gyeonggi-do, Republic of Korea), following to the manufacturer's instructions. NE-PER nuclear and cytoplasmic extraction reagents (Invitrogen) were applied for the preparation of mitochondrial and cytoplasmic proteins. After quantifying protein concentration, equal amounts of protein samples were separated to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then transferred into polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), as previously reported [35]. The membranes were subjected with specific primary antibodies at 4 °C overnight, and horseradish peroxidase-conjugated secondary antibodies for 2 h in sequence, and developed using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare Life Sciences, Little Chalfont, UK). Information on the antibodies used is provided in **Supplementary Table 1**. Protein expression was visualized by Fusion FX Image system. Quantitative analysis of mean pixel density was performed using the ImageJ® software (National Institutes of Health, Bethesda, MD, USA).

2.6 Caspase activity assay

Caspase colorimetric assay kits (R&D Systems, Minneapolis, MN, USA) were used to assess the activity of caspase-3, -8 and -9, according the manufacturer's protocol. Briefly, cell lysates were prepared after PD treatment, and then 20 μg of cell lysates in 100 μL of reaction buffer containing each caspase substrate provided in the kit were incubated at 37 °C for 2 h. Then, the absorbance was measured at 405 nm using a spectrophotometer, as previously described [36].

2.7 Detection of mitochondrial membrane potential (MMP) and ROS production

To evaluate the values of MMP, the cells were dyed with 10 μM of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Invitrogen) for 30 min, and MMP was detected *via* flow cytometry [37]. In order to measure the intracellular ROS, 5,6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA, Invitrogen) staining assay was applied as described previously. The stained cells were observed using flow cytometry and fluorescence microscope (Carl Zeiss) [38].

2.8 Statistical analysis

All data were obtained from at least three experiments. Data was presented as the mean ± standard deviation (SD), which was analyzed by variance *via* ANOVA-Tukey's post hoc test (version 5.03; GraphPad Prism Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

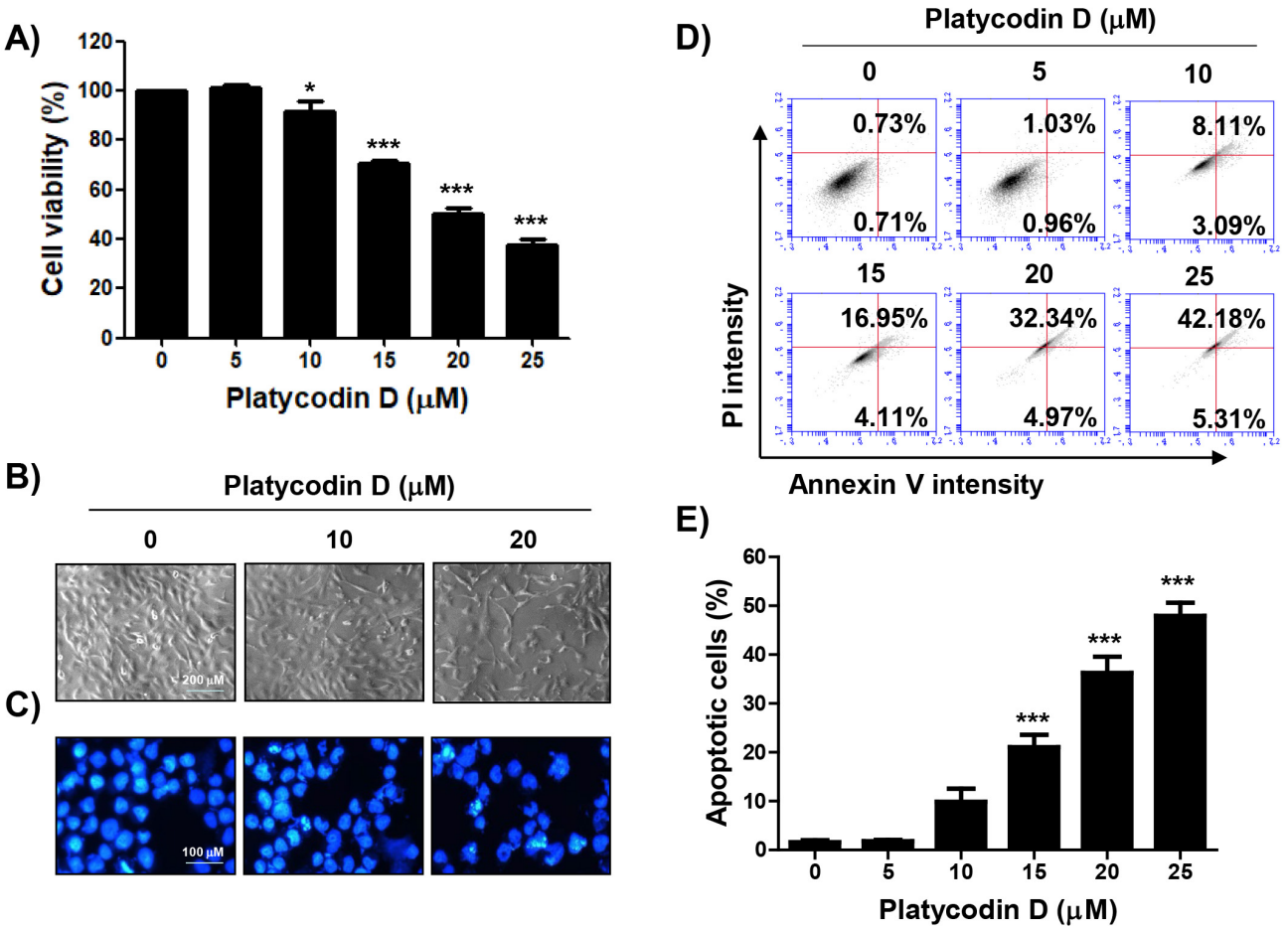

Fig. 1. Reduction of cell viability and induction of apoptosis by PD in PC3 cells. PC3 cells were treated with the indicated concentrations of PD for 48 h. (A) After treatment with PD, cell viability was measured by the MTT assay. (B) Morphological changes after treatment with PD were captured on a phase-contrast microscope. (C) The nuclear morphological changes were observed using DAPI staining, and then photographed under a fluorescence microscope. (D and E) The fixed cells were stained with annexin V/PI for flow cytometry analysis. (D) Representative flow cytometry plots are presented. (E) The populations of apoptotic cells were determined by expressing the numbers of annexin V^+ cells. The data are presented as the mean ± SD of three independent experiments ($^{**}P < 0.01$ and $^{***}P < 0.001$ compared to untreated cells).

3. Results

3.1 PD inhibits cell viability and induces apoptosis in PC3 cells

To investigate the cytotoxic effect of PD, an MTT assay was performed. Fig. 1A showed that PD significantly inhibited the viability of PC3 cells in a concentration-dependent manner. In addition, morphological changes were observed in PD-treated cells, in which the cytoplasm was condensed, rounded up, and separated from the substratum (Fig. 1B). Therefore, DAPI staining was conducted to examine if apoptosis is involved in the decrease in cell growth caused by PD. As shown in Fig. 1C. PD enhanced change to characteristic morphology of apoptotic nuclei and destruction of cell membrane in PC3 cells. Furthermore, PD-treated cells increased the number of apoptotic cells as indicated by the Annexin V/PI double staining assay results in a concentration-dependent manner (Fig. 1D,E).

3.2 PD regulates the levels of death receptor (DR)-associated and Bcl-2 family members in PC3 cells

It was next evaluated whether the DR-associated and Bcl-2 family proteins were involved in PD-induced apoptosis. As indicated in Fig. 2A and C, the expression of Fas, and Fas ligand (FasL) was markedly up-regulated in a concentration-dependent manner. Among the Bcl-2 family proteins, PD treatment led to an apparently up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2. In addition, the expression of Bid was suppressed, whereas the expression of truncated Bid (tBid) gradually increased (Fig. 2A,C). Meanwhile, in the case of the cellular FADD-like IL-1β-converting enzyme-inhibitory protein (c-FLIP), PD down-regulated the expression of short c-FLIP (c-$FLIP_S$) and long c-FLIP (c-$FLIP_L$) (Fig. 2A,C).

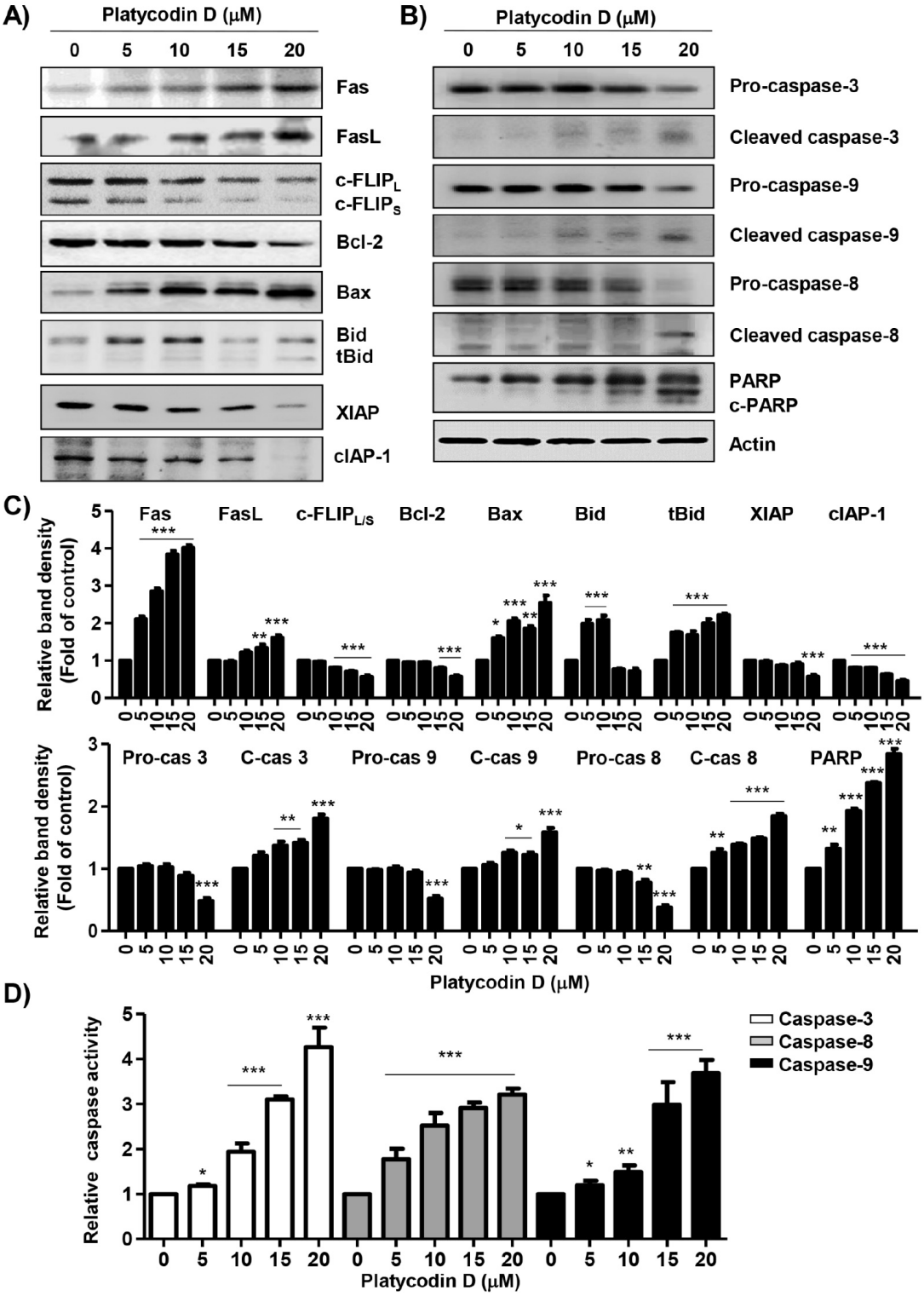

Fig. 2. The effects of PD on the expression of DR-related and Bcl-2 family members, and caspases in PC3 cells. PC3 cells were stimulated with different concentrations of PD for 48 h. (A and B) After treatment with the indicated concentrations of PD for 48 h, Western blotting was then performed using the indicated antibodies and an ECL detection system. Actin was used as an internal control. (C) The expression of each protein was indicated as fold change with control. (D) The activities of caspase-8, -9 and -3 were examined using colorimetric caspase assay kits. (C and D) Each bar indicated the mean ± SD of three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to untreated cells).

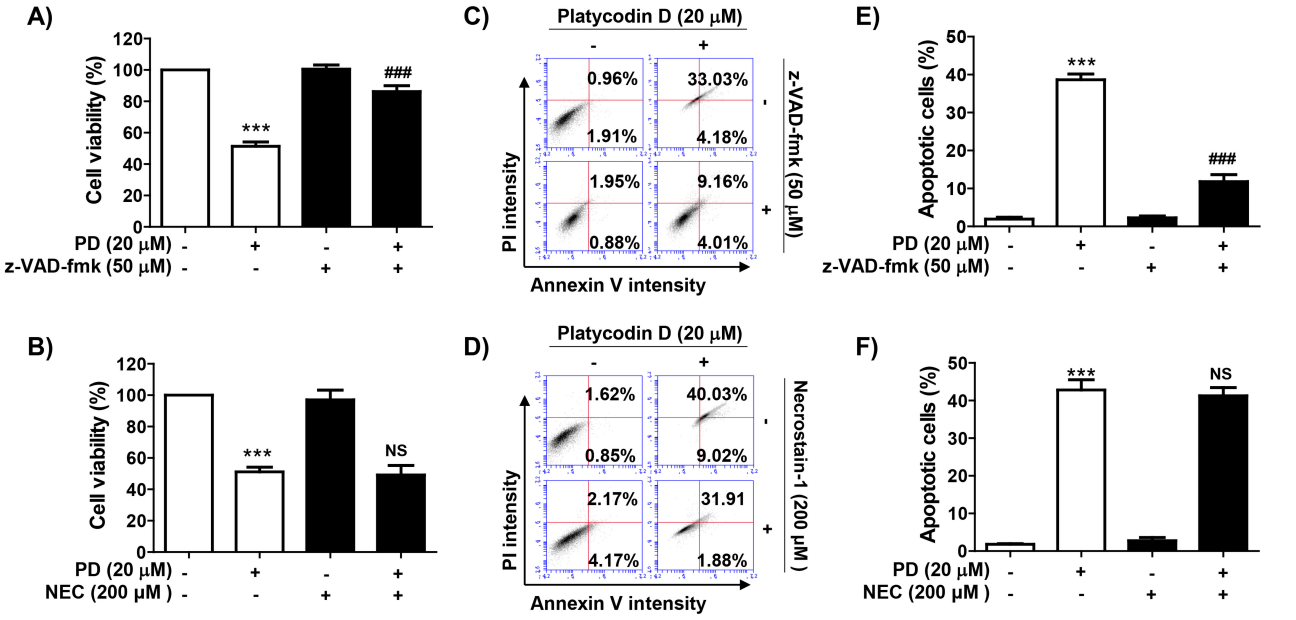

Fig. 3. The effects of caspase and necroptosis inhibitors on PD-mediated growth reduction and apoptosis in PC3 cells. The cells were pre-treated with 50 μM z-VAD-fmk, a pan-caspase inhibitor, or 200 μM necrostain-1 (NEC), a necroptosis inhibitor, for 1 h, and maintained in the presence or absence of 20 μM PD for 48 h. (A,B) Cell viability was measured by the MTT assay. (C–F) The fixed cells were stained with annexin V/PI for flow cytometry analysis. (C,D) Representative flow cytometry plots are presented. (E,F) The populations of apoptotic cells were determined by expressing the numbers of annexin V^+ cells. The data are presented as the mean $\pm$ SD of three independent experiments ($^{***}P < 0.001$ compared to control; $^{###}P < 0.001$ compared to PD-treated cells; NS, not significant compared to PD-treated cells).

3.3 PD activates caspases in PC3 cells

Since changes in the expression of DR-related and Bcl-2 family proteins were observed, it was investigated whether caspase activation was involved in apoptosis by PD. As shown in Fig. 2B and C, the expressions of both pro-caspase-8 and pro-caspase-9 were apparently reduced with increasing concentrations of PD. In addition, PD gradually up-regulated the expression of cleaved caspase-3, -8, and -9. PD also suppressed the expression of pro-caspase-3, a typical effector caspase that converges on both extrinsic and intrinsic pathways. Furthermore, cleaved form of poly (ADP-ribose) polymerase (PARP) was concentration-dependently up-regulated by treatment with PD, which was associated with down-regulation of inhibitor of apoptosis protein (IAP) family members including cIAP-1 and XIAP (Fig. 2B,C). In addition, PD significantly increased the activity of caspase-3, -8 and -9, which corresponded with the results of Western blot analysis (Fig. 2D). In order to confirm that PD-induced apoptosis was mediated by caspase-dependent pathways, the effect of PD on cell viability and apoptosis in pre-treatment of the pan-caspase inhibitor, z-VAD-fmk, was assessed. As shown in Fig. 3, PD-induced reduction in cell viability and induction of apoptosis were significantly reduced in the presence of z-VAD-fmk, whereas the necroptosis inhibitor, necrostain-1, had no effect on cell viability and apoptosis.

3.4 PD disrupts mitochondrial integrity in PC3 cells

To further evaluate the effect of PD on mitochondria-mediated intrinsic apoptosis in PC3 cells, the mitochondrial-function using JC-1 dye, an indicator of MMP, was assessed. Fig. 4A shows that PD significantly decreased the number of JC-1 aggregates, and concurrently increased JC-1 monomers in a concentration-dependent manner. In addition, PD gradually up-regulated the expression of cytosolic cytochrome *c*, whereas it down-regulated the expression of mitochondrial cytochrome *c* (Fig. 4B,C). This result suggested that PD induced release of cytochrome *c* from mitochondria by destruction of mitochondrial membrane intensity.

3.5 PD stimulates intracellular ROS generation of in PC3 cells

To investigate the effect of PD on intracellular ROS generation, DCF-DA staining was performed. As indicated in Fig. 4D and E, the accumulation of intracellular ROS was greatly increased within 1 h after treatment with PD However, PD-induced ROS level was significantly suppressed from 41.97% to 12.14% by the ROS scavenger NAC, based on flow cytometric analysis. In addition, fluorescence microscopy also demonstrated that PD markedly increased ROS production, which was mitigated by NAC treatment (Fig. 4F).

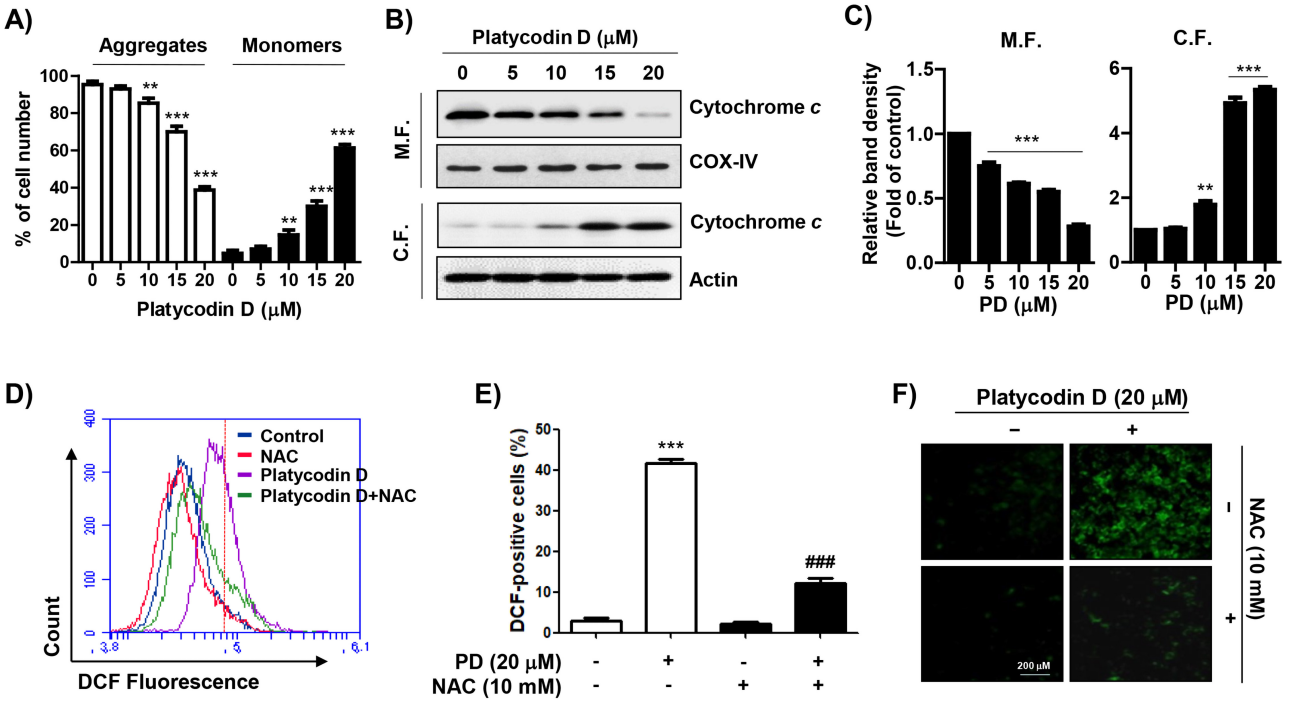

Fig. 4. Induction of mitochondrial dysfunction, cytosolic release of cytochrome *c* and ROS generation by PD in PC3 cells. (A) After treatment with PD, the cells were stained with JC-1 and then changes of MMP were determined by a flow cytometer. The data indicated the mean ± SD of three independent experiments ($^{**}P < 0.01$ and $^{***}P < 0.001$ compared to untreated cells). (B) Expression levels of cytochrome *c* using cytoplasmic and mitochondrial fractions were investigated by Western blot analysis. Actin and cytochrome oxidase subunit VI (COX VI) in each protein extract were presented as loading controls. (C) The expression of cytochrome *c* was indicated as fold change with control. Each bar indicated the mean ± SD of three independent experiments ($^{**}P < 0.01$ and $^{***}P < 0.001$ compared to untreated cells). (D–F) The cells were stimulated with 20 μM PD for 1 h or incubated with or without 20 μM PD for 1 h before treatment with 10 mM NAC for 1 h. (D and E) After removal of the medium, the cells were incubated with medium containing DCF-DA for 20 min. (D) The levels of intracellular ROS generation were determined by a flow cytometer. (E) The data represents the mean ± SD of three independent experiments (n = 6, $^{***}P < 0.001$ compared to control; $^{\#\#\#}P < 0.001$ compared to PD-treated cells). (F) Fluorescence images were taken with a fluorescence microscope. The fluorescent images were taken with a fluorescence microscope (200×).

3.6 PD-induced apoptosis is ROS-dependent in PC3 cells

To evaluate whether PD-induced apoptosis was associated with the production of ROS, it was evaluated the effect of NAC on loss of MMP induced by PD. The results showed that PD-induced decreases in JC-1 aggregates and increase in JC-1 monomers were significantly reversed by NAC (Fig. 5A). In addition, NAC markedly decreased PD-induced cytosolic release of cytochrome *c* (Fig. 5B,C). These results demonstrated that blocking ROS inhibited PD-induced destruction of mitochondrial integrity, and subsequently suppressed the release of cytochrome *c* from mitochondria into cytosol. Furthermore, NAC significantly attenuated the suppressed cell viability caused by PD and apoptosis induced by PD (Fig. 5D–F).

4. Discussion

In the present study, it was investigated whether PD induce apoptosis in human prostate cancer PC3 cells, and whether it can be considered a natural source of therapeutic for prostate cancer. According to the findings of this study, PD concentration-dependently induced cytotoxicity while inducing apoptosis in PC3 cells. Several previous studies demonstrated that PD showed minimal effect on normal cells including non-malignant human prostate epithelial cells. Zhou *et al.* [29]. reported that 50% growth inhibition concentration of PD on prostate epithelial RWPE-1 cells was over 50 μM. Furthermore, Chun *et al.* [39] showed that PD at the concentration up to 50 μM did not affect the cell viability of human normal cells lines such as lung fibroblast MRC-5, normal breast MCF-10A, and Chang liver cells. In addition, there has been reported that PD at the concentration up to 30 μM did not affect the survival of C2C12 myoblasts [13]. Moreover, the concentration of 20 μM PD did not exhibit any cytotoxicity effect on rat ventricular H9c2c cells [40]. Based on these finding, it was thought that PD has selective activity against malignant cells.

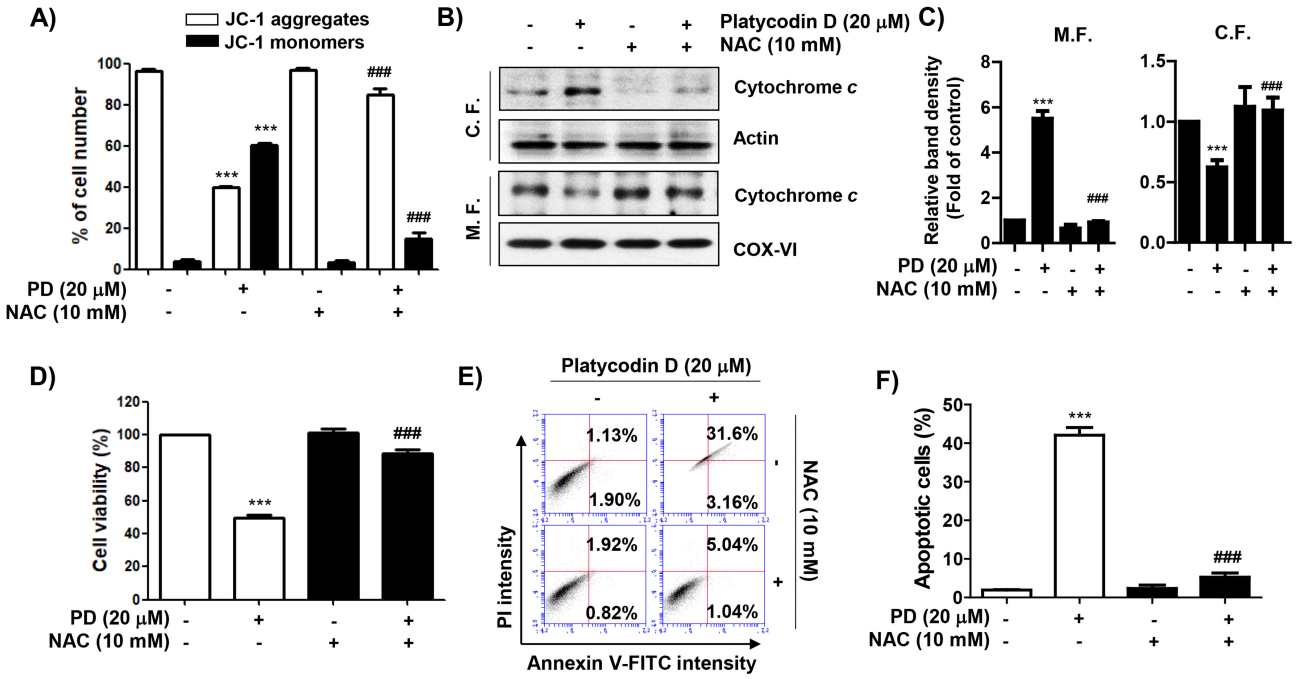

Fig. 5. The role of ROS on PD-induced cytotoxicity and apoptosis in PC3 cells. After pretreatment with 10 mM NAC for 1 h, and then 20 μM platycodin was added to the medium for another 48 h. (A) The changes of MMP were determined by a flow cytometer. (B) Cytoplasmic and mitochondrial fractions were used to examine cytochrome *c* expression. (C) The expression of cytochrome *c* was indicated as fold change with control. Each bar indicated the mean ± SD of three independent experiments ($^{***}P < 0.001$ compared to control; $^{###}P < 0.001$ compared to PD-treated cells). (D) Cell viability was measured the MTT assay. (E and F) The cells were fixed and stained with annexin V/PI for flow cytometry analysis. (E) Representative flow cytometry plots are presented. (F) The populations of apoptotic cells were determined by expressing the numbers of annexin V^+ cells. The data are presented as the mean ± SD of three independent experiments ($^{***}P < 0.001$ compared to control; $^{###}P < 0.001$ compared to PD-treated cells; NS, not significant compared to PD-treated cells).

Since understanding the mechanism of apoptosis is important in the pathogenesis of cancer, it was investigated whether apoptosis pathways were affected by PC3-induced cytotoxicity in PD. In general, apoptosis, a type of programmed cell death, is divided into either DR-initiated extrinsic or mitochondria-mediated intrinsic pathways [4,41]. The DR-mediated extrinsic pathway is initiated when the death ligands bind to the DRs, which triggers the activation of caspase-8, an inducing caspase of the extrinsic pathway. Thereafter, activation of caspase-8 leads to the activation of effector caspases such as caspase-3 and -7, which act as the final executor of apoptosis. In this process, c-FLIP acts as an important regulator of caspase-8 activation. c-FLIP suppresses DR-mediated apoptosis by blocking caspase-8 activation in death-inducing signaling complex (DISC) as it competes with pro-caspase-8 to bind to the Fas-associated death domain [1,3]. The present results showed that the expressions of Fas as well as its ligand FasL were concentration-dependently up-regulated by treatment with PD, similar to the result of a previous study using human gastric cancer cells [27]. Subsequently, PD down-regulated the expression of c-FLIP$_L$ and c-FLIP$_S$, and induced activation of caspase-8. These results indicated that PD-induced apoptosis derives from Fas/FasL interaction, and leads to activation of caspase-8 through down-regulation of c-FLIP, thus demonstrating that the extrinsic pathway is involved in PC3 cells. These results are consistent with Chun *et al.*'s finding [39]. They suggested that PD enhanced the expression of FasL protein and mRNA levels in AGS human gastric cancer cells. In addition, they demonstrated that PD mediated apoptosis by inducing both the synthesis and cell surface localization of Fas-L, which binds to Fas and activates caspase-8. In 2006, Ahn *et al.* [42] reported that PD upregulated the expression the Fas and the FasL in HaCaT cells, and ultimately involved in DNA fragmentation, caspase-3 and -8 activation.

On the other hand, the intrinsic pathway is initiated by the release of cytochrome *c* from the mitochondria to the cytoplasm. This pathway is tightly regulated by the Bcl-2 family members, which consist of anti-apoptotic and pro-apoptotic proteins [2,41]. In this study, we verified that PD led to up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2, activated caspase-9 and -3, and induced the cleavage of PARP, a major substrate protein of activated caspase-3 [1,3]. Similar to the finding, several previous studies demonstrated that PD-induced

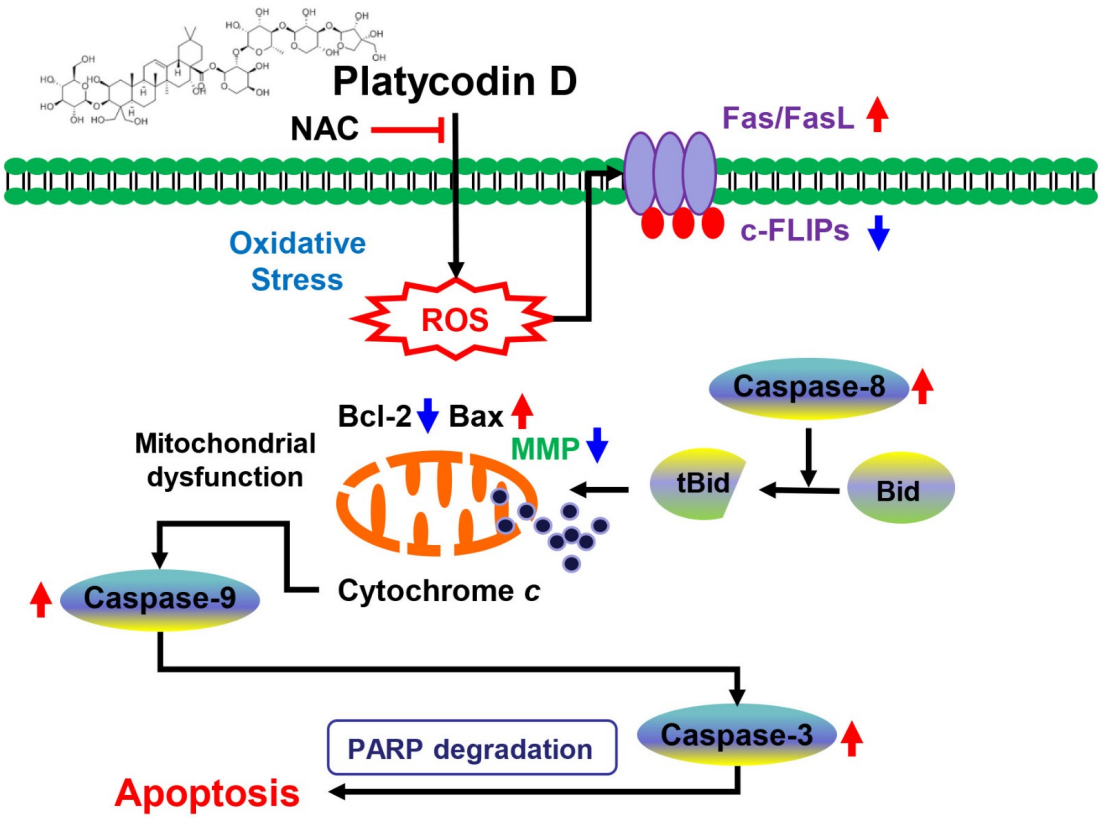

Fig. 6. Proposed model for PD-induced apoptosis in PC3 human prostate cancer cells.

apoptosis has been associated with the intrinsic pathway *via* mitochondrial Bcl-2 family members in AGS gastric cancer cells, HepG2 hepatoma cancer cells, MCF-7 breast cancer cells, and glioma U251 cells [25,31,39,43]. More recently, there has been reported that PD induced apoptosis through inducing of mitochondrial-mediated apoptotic proteins including Bas, cytochrome *c*, cleaved caspase-3, and eventually lead to alleviates liver fibrosis in hepatic stellate cells [44]. Furthermore, PD up-regulated tBid expression with down-regulation of total Bid expression. Truncation of Bid for the production of tBid is caused by activated caspase-8, and tBid oligomerizes in the mitochondrial outer membrane to cause mitochondrial dysfunction. As a result, the loss of MMP results in the release of cytochrome *c*, which serves to connect and amplify the two apoptotic pathways [4,5]. The present results demonstrated that PD destroyed the integrity of mitochondria as a result of loss of MMP, which contributed to cytosolic release of cytochrome *c*. However, PD-induced cytotoxicity and apoptosis in PC3 cells were significantly suppressed by pretreatment with pan-caspase inhibitor. Therefore, these results indicated that both caspase-dependent extrinsic and intrinsic pathways might be due to the regulation of Bcl-2 family proteins in PC3 cells.

Mitochondria are the primary cellular source of ROS generation, and oxidizing mitochondrial pores lead to an increase of ROS levels [1,45]. Increasing cellular ROS levels accelerates the oxidation of DNA, proteins and lipids, thus leading to cell death by cellular dysfunction [46,47]. In this regard, recent studies have shown that these biochemical properties of cancer cells can be exploited for therapeutic benefits, in contrast to the tumor-promoting effects of increased ROS [23]. Therefore, the effects of PD on the production of ROS were further evaluated. The results showed that PD significantly increased ROS production, but PD-induced ROS accumulation was markedly attenuated by NAC, the ROS scavenger. In addition, PD-induced apoptotic events, including loss of MMP, cytosolic release of cytochrome *c* and cytotoxicity, were all fully blocked by NAC. These results demonstrated that PD-mediated apoptosis was markedly attenuated when ROS generation was artificially blocked by NAC. Therefore, apoptosis of PC3 cells which was induced by PD was clearly ROS-dependent. These results are consistent with previous studies in various carcinoma cells. For example, Yu *et al.* [31] suggested that PD induced ROS-mediated apoptosis and endoplasmic reticulum stress response in MCF-1 breast cells, and Shin *et al.* [32] also reported that PD exhibited ROS-induced apoptosis in U937 leukemia cells. Moreover, there has been reported that PD induced apoptosis of PC-12 pheochromocytoma cells through a ROS-mediated mitochondrial dysfunction pathway. Additionally, a previous report demonstrated that PD have protective effect against ROS-induced DNA damage and apoptosis in C2C12 my-

oblasts [13]. Over all, the current results show that PD-induced apoptosis mediated Fas/FasL interaction, and activation of caspase-8 through down-regulation of c-FLIP. In addition, PD induced mitochondrial dysfunction through modulation of Bcl-2 family proteins (Fig. 6). Meanwhile, PD-induced apoptosis in PC3 cells was simultaneously mediated by caspase-dependent intrinsic and extrinsic pathways, which was ROS dependent.

5. Conclusions

Taken together, the present finding provided persuasive evidence for promising role of PD in treatment of prostate cancer. It has been shown that PD induced apoptosis through ROS-dependently intrinsic and extrinsic pathway in PC3 cells. However, although this finding indicate that PD can be applied as an effective treatment for prostate cancer, it is necessary to confirm the anti-cancer efficacy of PD through an experimental model of prostate cancer, and further studies on the clinical application of PD are warranted.

Author contributions

YHC is the sole contributor to this article.

Ethics approval and consent to participate

Not applicable.

Acknowledgment

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Conflict of interest

The author declares no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://doi.org/10.31083/jomh.2021.132.

References

[1] Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and molecular targeting therapy in cancer. BioMed Research International. 2014; 2014: 150845.

[2] Schnekenburger M, Dicato M, Diederich M. Plant-derived epigenetic modulators for cancer treatment and prevention. Biotechnology Advances. 2014; 32: 1123–1132.

[3] Kiraz Y, Adan A, Kartal Yandim M, Baran Y. Major apoptotic mechanisms and genes involved in apoptosis. Tumor Biology. 2016; 37: 8471–8486.

[4] Pfeffer CM, Singh ATK. Apoptosis: a Target for Anticancer Therapy. International Journal of Molecular Sciences. 2018; 19: 448.

[5] Fouad YA, Aanei C. Revisiting the hallmarks of cancer. American Journal of Cancer Research. 2017; 7: 1016–1036.

[6] Medema RH, Macůrek L. Checkpoint control and cancer. Oncogene. 2012; 31: 2601–2613.

[7] Ranjan A, Ramachandran S, Gupta N, Kaushik I, Wright S, Srivastava S, *et al.* Role of phytochemicals in cancer prevention. International Journal of Molecular Sciences. 2019; 20: E4981.

[8] Park M, Park SY, Lee HJ, Kim CE. A Systems-level analysis of mechanisms of *Platycodon grandiflorum* based on a network harmacological approach. Molecules. 2018; 23: E2841.

[9] Zhang L, Wang Y, Yang D, Zhang C, Zhang N, Li M, *et al*. Platycodon grandiflorus - an ethnopharmacological, phytochemical and pharmacological review. Journal of Ethnopharmacology. 2015; 164: 147–161.

[10] Nyakudya E, Jeong JH, Lee NK, Jeong Y. Platycosides from the Roots of Platycodon grandiflorum and their Health Benefits. Preventive Nutrition and Food Science. 2014; 19: 59–68.

[11] Xie Y, Sun H, Li D. Platycodin D is a potent adjuvant of specific cellular and humoral immune responses against recombinant hepatitis B antigen. Vaccine. 2009; 27: 757–764.

[12] Zhang J, Song N, Liu Y, Guo J. Platycodin D inhibits beta-amyloid-induced inflammation and oxidative stress in BV-2 cells *via* suppressing TLR4/NF-kappaB signaling pathway and activating Nrf2/HO-1 signaling pathway. Neurochemical Research. 2021; 46: 638–647.

[13] Choi YH. Activation of the Nrf2/HO-1 signaling pathway contributes to the protective effects of platycodin D against oxidative stress-induced DNA damage and apoptosis in C2C12 myoblasts. General Physiology and Biophysics. 2020; 39: 519–530.

[14] Wang G, Guo H, Wang X. Platycodin D protects cortical neurons against oxygen-glucose deprivation/reperfusion in neonatal hypoxic-ischemic encephalopathy. Journal of Cellular Biochemistry. 2019; 120: 14028–14034.

[15] Ye Y, Pei L, Ding J, Wu C, Sun C, Liu S. Effects of Platycodin D on S100a8/a9-induced inflammatory response in murine mammary carcinoma 4T1 cells. International Immunopharmacology. 2019; 67: 239–247.

[16] Lee EG, Kim KH, Hur J, Kang JY, Lee HY, Lee SY. Platycodin D attenuates airway inflammation *via* suppression Th2 transcription factor in a murine model of acute asthma. Journal of Asthma. 2021; 23: 1–11.

[17] Fu C, Liu Y, Leng J, Zhang J, He Y, Chen C, *et al*. Platycodin D protects acetaminophen-induced hepatotoxicity by inhibiting hepatocyte MAPK pathway and apoptosis in C57BL/6J mice. Biomedicine & Pharmacotherapy. 2018; 107: 867–877.

[18] Hwang YP, Choi JH, Kim HG, Khanal T, Song GY, Nam MS, *et al*. Saponins, especially platycodin D, from Platycodon grandiflorum modulate hepatic lipogenesis in high-fat diet-fed rats and high glucose-exposed HepG2 cells. Toxicology and Applied Pharmacology. 2013; 267: 174–183.

[19] Kim H, Park J, Jung Y, Ahn KS, Um J. Platycodin D, a novel activator of AMP-activated protein kinase, attenuates obesity in db/db mice via regulation of adipogenesis and thermogenesis. Phytomedicine. 2019; 52: 254–263.

[20] Lin Y, Lin Y, Kuo W, Shen C, Cheng Y, Lin Y, *et al*. Platycodin D Reverses Pathological Cardiac Hypertrophy and Fibrosis in Spontaneously Hypertensive Rats. The American Journal of Chinese Medicine. 2018; 46: 537–549.

[21] Xie Y, Sun H, Li D. Platycodin D Improves the Immunogenicity of Newcastle Disease Virus-Based Recombinant Avian Influenza Vaccine in Mice. Chemistry & Biodiversity. 2010; 7: 677–689.

[22] Chen D, Chen T, Guo Y, Wang C, Dong L, Lu C. Suppressive effect of platycodin D on bladder cancer through microRNA-129-5p-mediated PABPC1/PI3K/AKT axis inactivation. The Brazilian Journal of Medical and Biological Research. 2021; 54: e10222.

[23] Khan M, Maryam A, Zhang H, Mehmood T, Ma T. Killing cancer with platycodin D through multiple mechanisms. Journal of Cellular and Molecular Medicine. 2016; 20: 389–402.
[24] Zhang X, Zhai T, Hei Z, Zhou D, Jin L, Han C, Wang J. Effects of platycodin D on apoptosis, migration, invasion and cell cycle arrest of gallbladder cancer cells. Oncology Letters. 2020; 20: 311.
[25] Li T, Xu W, Wu G, Chen X, Wang Y, Lu J. Platycodin D induces apoptosis, and inhibits adhesion, migration and invasion in HepG2 hepatocellular carcinoma cells. Asian Pacific Journal of Cancer Prevention. 2014; 15: 1745–1749.
[26] Li W, Tian Y, Liu Y, Wang Z, Tang S, Zhang J, *et al*. Platycodin D exerts anti-tumor efficacy in H22 tumor-bearing mice via improving immune function and inducing apoptosis. The Journal of Toxicological Sciences. 2016; 41: 417–428.
[27] Chun J, Kim YS. Platycodin D inhibits migration, invasion, and growth of MDA-MB-231 human breast cancer cells via suppression of EGFR-mediated Akt and MAPK pathways. Chemico-Biological Interactions. 2013; 205: 212–221.
[28] Seo Y, Kang O, Kong R, Zhou T, Kim S, Ryu S, *et al*. Polygalacin D induces apoptosis and cell cycle arrest via the PI3K/Akt pathway in non-small cell lung cancer. Oncology Reports. 2018; 39: 1702–1710.
[29] Zhou R, Lu Z, Liu K, Guo J, Liu J, Zhou Y, *et al*. Platycodin D induces tumor growth arrest by activating FOXO3a expression in prostate cancer *in vitro* and *in vivo*. Current Cancer Drug Targets. 2015; 14: 860–871.
[30] Zeng C, Zhang C, Yao J, Lai S, Han B, Li W, *et al*. Platycodin D induced apoptosis and autophagy in PC-12 cells through mitochondrial dysfunction pathway. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2016; 168: 199–205.
[31] Yu JS, Kim AK. Platycodin D induces reactive oxygen species-mediated apoptosis signal-regulating kinase 1 activation and endoplasmic reticulum stress response in human breast cancer cells. Journal of Medicinal Food. 2012; 15: 691–699.
[32] Shin DY, Kim GY, Li W, Choi BT, Kim ND, Kang HS, *et al*. Implication of intracellular ROS formation, caspase-3 activation and Egr-1 induction in platycodon D-induced apoptosis of U937 human leukemia cells. Biomedicine & Pharmacotherapy. 2009; 63: 86–94.
[33] Sirirattanakul S, Santiyanont R. *Fimbristylis ovata* extract and its ability to encounter AGEs-induced neurotoxicity in SH-SY5Y. Toxicology Research. 2021; 37: 355–367.
[34] Ojima T, Kawami M, Yumoto R, Takano M. Differential mechanisms underlying methotrexate-induced cell death and epithelial-mesenchymal transition in A549 cells. Toxicology Research. 2021; 37: 293–300.
[35] Choi YH. Trans-cinnamaldehyde protects C2C12 myoblasts from DNA damage, mitochondrial dysfunction and apoptosis caused by oxidative stress through inhibiting ROS production. Genes & Genomics. 2021; 43: 303–312.
[36] Liang Y, Kong D, Zhang Y, Li S, Li Y, Ramamoorthy A, *et al*. Fisetin Inhibits Cell Proliferation and Induces Apoptosis via JAK/STAT3 Signaling Pathways in Human Thyroid TPC 1 Cancer Cells. Biotechnology and Bioprocess Engineering. 2020; 25: 197–205.
[37] Bae C, Lee C, Ahn T. Encapsulation of Apoptotic Proteins in Lipid Nanoparticles to Induce Death of Cancer Cells. Biotechnology and Bioprocess Engineering. 2020; 25: 264–271.
[38] Pham TNA, Le B, Yang SH. Anticancer Activity of the Potential Pyropia yezoensis Galactan Fractionated in Human Prostate Cancer Cells. Biotechnology and Bioprocess Engineering. 2021; 26: 63–70.
[39] Chun J, Joo EJ, Kang M, Kim YS. Platycodin D induces anoikis and caspase-mediated apoptosis via p38 MAPK in AGS human gastric cancer cells. Journal of Cellular Biochemistry. 2013; 114: 456–470.
[40] Wang Y, Che J, Zhao H, Tang J, Shi G. Platycodin D inhibits oxidative stress and apoptosis in H9c2 cardiomyocytes following hypoxia/reoxygenation injury. Biochemical and Biophysical Research Communications. 2018; 503: 3219–3224.
[41] Schultz DR, Harrington WJ. Apoptosis: programmed cell death at a molecular level. Seminars in Arthritis and Rheumatism. 2003; 32: 345–369.
[42] Ahn KS, Hahn B, Kwack K, Lee EB, Kim YS. Platycodin D-induced apoptosis through nuclear factor-kappaB activation in immortalized keratinocytes. European Journal of Pharmacology. 2006; 537: 1–11.
[43] Xu C, Sun G, Yuan G, Wang R, Sun X. Effects of platycodin D on proliferation, apoptosis and PI3K/Akt signal pathway of human glioma U251 cells. Molecules. 2014; 19: 21411–21423.
[44] Liu Y, Cong S, Cheng Z, Hu Y, Lei Y, Zhu L, *et al*. Platycodin D alleviates liver fibrosis and activation of hepatic stellate cells by regulating JNK/c-JUN signal pathway. European Journal of Pharmacology. 2020; 876: 172946.
[45] Badrinath N, Yoo SY. Mitochondria in cancer: in the aspects of tumorigenesis and targeted therapy. Carcinogenesis. 2018; 39: 1419–1430.
[46] Moloney JN, Cotter TG. ROS signalling in the biology of cancer. Seminars in Cell & Developmental Biology. 2018; 80: 50–64.
[47] Galadari S, Rahman A, Pallichankandy S, Thayyullathil F. Reactive oxygen species and cancer paradox: to promote or to suppress? Free Radical Biology & Medicine. 2017; 104: 144–164.