Original article

THAI BULLETIN OF PHARMACEUTICAL SCIENCES
ISSN 2586-8659 (online)

PINOCEMBRIN ATTENUATES COLISTIN-INDUCED HUMAN RENAL PROXIMAL TUBULAR CELL APOPTOSIS

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ABSTRACT

Colistin is an essential last-resort polypeptide antibiotic widely used for treatment of multidrug-resistant (MDR) caused by the emergence of gram-negative bacterial infection. However, the adverse effect of colistin has been associated with nephrotoxicity. The nephrotoxicity is mediated by excessive production of reactive oxygen species (ROS) and mitochondria damage in renal cells. Pinocembrin, one of the dominant bioactive compounds extracted from *Boesenbergia rotunda*, presents antioxidative properties and preventive role against mitochondrial dysfunction. Thus, this study aimed to investigate the renoprotective effects and cellular mechanisms of pinocembrin against colistin-induced renal proximal tubular cells toxicity. The results revealed that colistin treatment significantly reduced cell viability and enhanced apoptosis of human renal proximal tubular cells, RPTEC/TERT1 cells, compared with vehicle. These effects were attenuated when co-treated with pinocembrin. Moreover, colistin-activated cytotoxicity including ROS generation, loss of membrane potential and upregulation of apoptotic proteins expression such as cytochrome C and caspase-3 were suppressed in pinocembrin treatment. Therefore, pinocembrin exerts protective effects against human renal proximal tubular cells apoptosis by ameliorated colistin-induced mitochondrial impairment.

Keywords: colistin, pinocembrin, renal proximal tubular cells, mitochondrial dysfunction, renoprotection

Received: 5 February 2021; Revised: 19 March 2021; Accepted: 22 March 2021

Introduction

Acute kidney injury (AKI) due to drug toxicity has let to serious health complications in the current clinical situations. The pathophysiologic mechanism of drug-initiated nephrotoxicity is characterized by the alteration of intraglomerular hemodynamics, inflammation, crystal nephropathy, and tubular cell cytotoxicity.[1] Up to 60% of AKI patients, the diseases were developed by drug administration particularly antibiotics and conventional nonselective nonsteroidal anti-inflammatory drugs (NSAIDs).[2] One of the considerable antibiotics that leads to nephrotoxicity is colistin (polymyxin E). Colistin was known as an appreciable lipopeptide antibiotics in the family of polymyxins which is used as a last-line treatment against multidrug-resistant (MDR) caused by gram-negative bacterial infection.[3] The antibacterial activity of colistin have been reported to enhance permeability of the bacterial cell wall leading to cell lysis and death.[4] In addition, colistin has been awarded as the "Highest Priority Critically Important Antimicrobials" by The World Health Organization (WHO, 2019) which is achieve in therapeutic treatment for serious multi-resistant infections of *Enterobactericeae* and *Pseudomonas aeruginosa*.

Although the clinical effectiveness of colistin in antibiotic action is generally reported, the use of this drug has largely been associated with nephrotoxicity.[5,6] Colistin accumulates in the renal cortex regions which brings about mitochondria damage in the renal cells.[7] In addition, clinical manifestations of colistin were classified as reducing in creatinine clearance, cylindruria (urinary casts occurring) as well as oliguria (low urine output). These defects are also mentioned in animal models, like the previous study, administration of colistin for 7 days promotes epithelial cell vacuolation, tubular dilation and tubular necrosis.[8] Furthermore, colistin intraperitoneally injection for 6 days induces oxidative stress biomarkers including malondialdehyde and 8-hydroxydeoxyguanosine (8-OHdG) activating inflammation and tubular degeneration in rats.[6] However, the intracellular mechanism underlying colistin-triggered renal injury remains unclear, it suggests being related to the production of oxidative stress, activation of apoptosis pathway and promoting DNA adducts.[9] Thus, searching for novel nephroprotective agents to prevent colistin-induced nephrotoxicity is very challenging to minimize adverse effect of colistin treatment in clinical trials.

Pinocembrin or 5,7-dihydroxyflavanone is an abundant flavanone bioactive compounds isolated from a fingerroot ginger species, *Boesenbergia rotunda* (L.) Mansf. *(syn. B. pandurata (Roxb.) Schltr.),* which generally found in Southeast Asia, Sri Lanka and Southern China. Due to its several pharmacological activities including antioxidant, anti-inflammatory, anti-cancer and anti-bacterial effects[10], this plant is also acknowledged as a traditional medicinal plant. Moreover, *B. rotunda* contains several types of secondary metabolites and prenylated flavonoids which have been reported to suppress oxidative damage generated by reactive intermediates and attenuate intracellular ROS formation in HepG2 cells (human liver cancer cells).[11] Apart from antioxidant activity, in animal model, the coadministration of pinocembrin also reduces allergic airway inflammation regulated through suppression of Nuclear factor-κB (NF-κB) and cytokines interleukin (IL)-4, IL-5 and IL-13 in lung tissue of ovalbumin (OVA)-sensitized mice.[12]

Even though the high potency of this bioactive compound has been investigated, the protective effect and mechanism associated with colistin-induced renal cell damage or nephrotoxicity has never been elucidated. Therefore, the present study aimed to determine whether pinocembrin act as a nephroprotective agent to ameliorate renal cell damage caused by colistin treatment.

Materials and methods

Chemicals

Pinocembrin (purity >95%) was extracted from *Boesenbergia rotunda*. Colistin sulfate salt (15,000 units/mg, trypan blue, 2,7-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (MO, USA). Kit for evaluating cell apoptosis (Annexin V-FITC) was purchased from BD biosciences (CA, USA). Antibodies including Bcl-2, cytochrome C, and β-actin were obtained from Cell Signaling Technology Company (MA, USA). All other chemicals were analytic grade.

Cell culture

RPTEC/TERT1 cells (human renal proximal tubular epithelial cell line) were purchased from American Type Culture Collection (ATCC). RPTEC/TERT1 cells were cultured in the mixture of Dulbecco's modified Eagle's medium with Ham's F-12 medium supplemented with 1% penicillin/ streptomycin, 5 µg/ml human transferrin, 10 ng/ml recombinant human EGF, 25 ng/ml hydrocortisone, 0.05 mg/ml selenium and 5 µg/ml insulin. Cells were incubated at 37ºC under 5% CO_2 in 95% humidity. Cells were grown until 100% cell confluence in culture plate before further subsequent experimental procedures.

Cell viability assay

Cell viability was assessed following trypan blue exclusion assay. Briefly, RPTEC/TERT1 cells were plated into 96-well plates for 2 weeks at a density of 10^4 cells/well. After treatment, cells were discharged and stained with 0.4% trypan blue solution in buffer isotonic salt followed by observing under light microscope. Live and dead cells were monitored as unstained and stained cells respectively.

Assessment of cell apoptosis

Detection of live and dead cells were performed using apoptosis detection kit (annexin V-FITC/propidium iodide (PI)) following flow cytometry assay. After 48 h treatment, cells were harvested and stained with 5 µl of each Annexin V-FITC and PI for 15 min in darkness. Thereafter, cells were added with 400 µl of 1X binding buffer followed by flow cytometry analysis (BD Biosciences, CA, USA). The results were demonstrated based on the random sampling of 30,000 cells indicating as the percentage of live cells, apoptosis cells and necrosis cells.

Assessment of intracellular ROS detection

The level of intracellular ROS was measured using DCFH-DA fluorescent dye. RPTEC/TERT1 cells were seeded in 96-back well plate until 100% confluence. After 24 h treatments, cells were washed with DPBS. Thereafter, 10 µM DCFH-DA was added into the medium and incubated at 37 °C, 5% CO_2 in darkness for 30 min. Removing the dye followed by washed with DPBS three times and examined with fluorescence microscope (20X). The fluorescence absorbance was measured using the fluorescence plate reader (Operetta and EnVision, Perkin Elmer) with excitation and emission wavelengths of 480 and 530 nm, respectively. The levels of relative ROS were analyzed as percentage normalized with control.

Assessment of the alteration in mitochondrial membrane potential (MMP; $\Delta\psi_m$)

The alterations in MMP in the cells were determined using JC-1 fluorescence reagent. Cells were seeded into 96-back well plate until 100% confluence. After 24 h treatment, cells were treated with 20 µM JC-1 in DPBS at 37 ºC for 15 min. Removing the dye and washed with DPBS followed by detecting under fluorescence microscope with excitation wavelength of 488 nm. Monomeric form of JC-1 was emitted green fluorescence at 530 nm whereas aggregates form of JC-1 was emitted red fluorescence at 595 nm. The ratios of fluorescence intensity between red and green channels were interpreted as the mitochondrial membrane potential analyzing via image J software.

Assessment of protein expressions

The expression of protein related to apoptosis including cytochrome C and Bcl-2 were determined. Following 24 h treatment, the cellular proteins of RPTEC/TERT1 cells were extracted via 100 µl/well of lysis buffer. Lysates were centrifuge at 13,000 xg for 20 minutes at 4°C, and the supernatant was collected and stored at -80°C prior to electrophoresis.

Subsequently, western blot analysis was then performed. Protein samples were separated by 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes. The membrane was blocked with blocking solution containing nonfat dry milk (5%) in Tris-buffered Saline-Tween 20 (TBST) for 2 h and then incubation with primary antibodies including anti-cytochrome C, anti-Bcl-2 and anti-β actin at 4°C overnight. The membranes were washed and incubated with secondary antibody for 1.5 h at room temperature followed by immune complexes detection using Electro-Chemi-Luminescence (ECL). The blotting was exposed to UltraCruz autoradiography films (Santa Cruz, CA, USA) and the protein bands were analyzed and quantified using Image J software.

Data analysis and statistical methods

All data are given as the means ± SEM. The statistical significance was performed by one-way analysis of variance (ANOVA) followed by Tukey test for multigroup comparisons. Figures were prepared using GraphPad Prism 5.0 (GraphPad Software, CA, USA). p-value < 0.05 was considered significant difference.

Results

Pinocembrin ameliorates colistin-induced cytotoxicity in renal proximal tubular cells

To determine the protective effect of pinocembrin against cytotoxicity induced by colistin, we determined the effect of pinocembrin on viability of RPTEC/TERT1 cells under 200 µg/ml colistin treatment for 72 h. This condition has been proved to be the most appropriate concentration and time to initiate the toxicity based on IC_{50} value (data not shown). Colisin and pinocembrin were dissolved in the culture medium and DMSO (final concentration of DMSO <0.2% v/v with no observable toxic effects to cells), respectively. According to Fig. 1A, there was a significant decrease in RPTECT/TERT1 cell viability after colistin treatment compared with vehicle-treated cells. Co-treatment the cells with 25, 50 and 100 µM pinocembrin significantly attenuated the cytotoxic effect of colistin. Importantly, 50 µM pinocembrin was maximally promoted protective effect against colistin-induced cytotoxicity. Supporting to this data, co-treatment of colistin with 50 µM of pinocembrin presented more cell survival when compared with colistin-treated alone as shown in trypan blue-stained images (Fig. 1B).

Pinocembrin attenuates colistin-induced renal cell apoptosis

To tested whether attenuating effect of pinocembrin correlated by suppression of renal cell death, the effect of pinocembrin on colistin-induced cell death was determined. The results demonstrated that 200 µg/ml colistin was significantly increase apoptosis cell death as shown in flow cytometry dot-plot compared with control. Co-treatment the cells with colistin with pinocembrin at 50 µM partially reduced the percentage of cell apoptosis compared with colistin-treated cells (Fig. 2). Therefore, these results suggested that pinocembrin can ameliorate the apoptosis effect induced by colistin in RPTEC/TERT1 cells.

Pinocembrin suppresses mitochondrial damage activated by colistin

Activation of ROS leading to mitochondrial damage has been reported as a consequence of colistin treatment.[13] To determine whether pinocembrin can decrease ROS level initiated by colistin, the cellular levels of ROS were measured using DCFH-DA staining assay. As shown in Fig. 3A, colistin significantly promoted higher cellular fluorescence intensity which indicated high level of ROS compared with control. Reduction of fluorescence intensity was achieved when co-treated with 50 µM pinocembrin. Furthermore, accumulation of ROS initiated by colistin results in mitochondrial damage and reduce membrane potential.[14] Thereafter, the effect of pinocembrin on the potential changed of mitochondrial membrane was examined by using red/green fluorescence intensity. Colistin induced loss of membrane potential, represented as an increase in green fluorescence intensity compared with control. Co-treatment with 50 μM pinocembrin attenuated the effect of colistin-induced loss of membrane potential by increased the red/green fluorescence ratio (Fig 3B). Therefore, these results suggested that pinocembrin can reduce mitochondrial impairment triggered by colistin in RPTEC/TERT1 cells.

Pinocembrin attenuates protein expression of cytochrome C and cleaved caspase-3 whereas up-regulated Bcl-2

Activating protein expressions of cell apoptosis including cytochrome C and cleaved caspase-3 have previously reported following colistin treatment.[15] Thus, we next determined the effect of pinocembrin on colistin-induced apoptosis in renal proximal tubular cells. As demonstrated in Fig. 4, after treatment for 48 h, colistin markedly reduced the expression of anti-apoptosis protein, Bcl-2, together with increased cytochrome C and cleaved caspase-3 levels compared with vehicle-treated cells. Co-treatment of colistin with 50 μM pinocembrin significantly reversed the level of Bcl-2 protein and attenuated the expression of cytochrome C and cleaved-caspase 3 proteins. Pinocembrin alone did not significantly affect expression of these proteins.

Discussion

Colistin is a last-resort antibiotic agent beneficial for the treatment of infections that cannot be resolved with other antibiotic agents. Nevertheless, this drug causes nephrotoxicity as a major adverse effect with renal proximal tubular cell deaths being an important pathogenic event.[5] Here, we have identified pinocembrin as an anti-apoptotic agent alleviating colistin-induced apoptosis of human renal proximal tubular (RPTEC/TERT1) cells. These effects were associated with reversal of pro-apoptotic protein cytochrome C, cleaved caspase-3, and anti-apoptotic protein Bcl-2. In addition, pinocembrin reduced ROS generation and mitochondrial permeability induced by colistin.

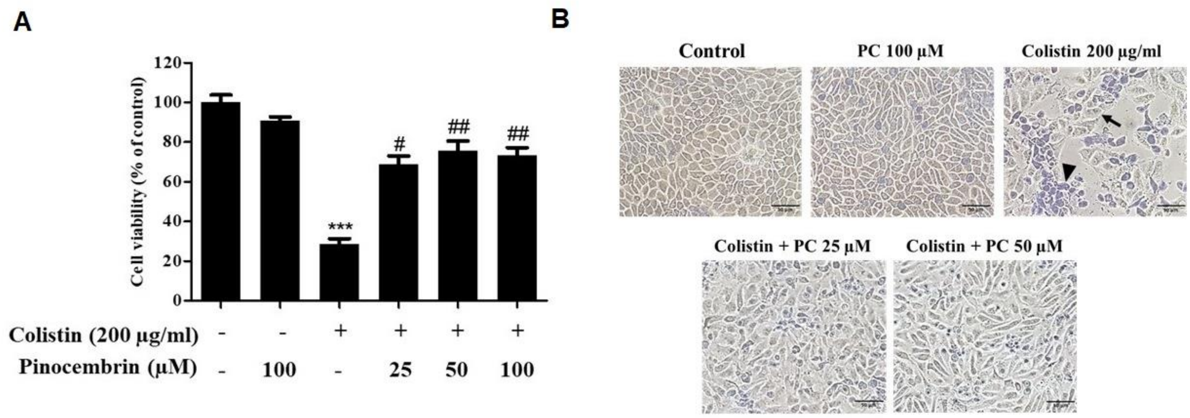

Figure 1 Protective effect of pinocembrin against colistin-induced cytotoxicity in RPTEC/TERT1 cell. (A) Data are presented as mean ± SEM of % control from four independent experiments. $^{***}p$ < 0.001 compared with control whereas $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01 compared with colistin-treated group. (B) Trypan blue staining images represent live cells (arrow) and dead cells (triangle) after colistin and pinocembrin treatment. PC, pinocembrin.

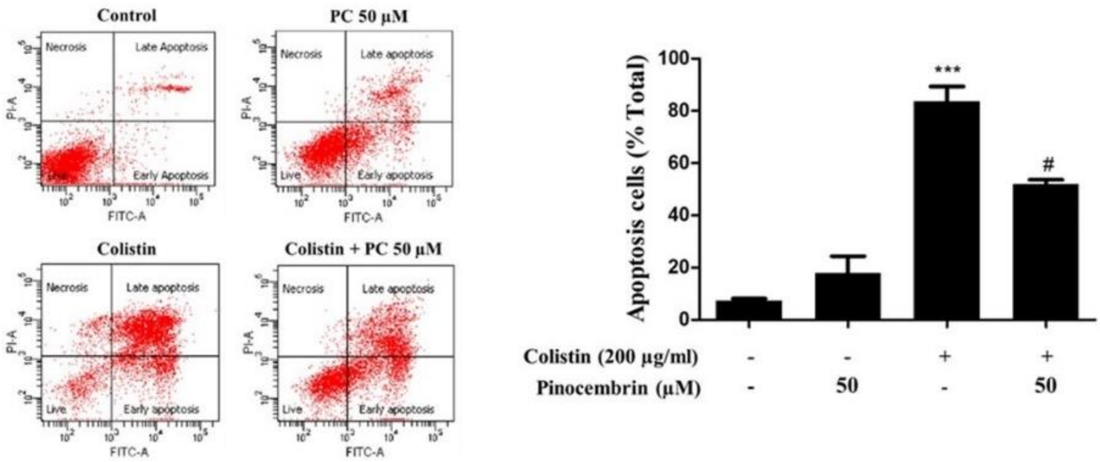

Figure 2 Protective effect of pinocembrin against colistin-induced apoptosis in RPTEC/TERT1 cells. Cells were treated with vehicle, pinocembrin alone or combined with 200 µg/ml colistin for 48 h. Flow cytometry dot plots showing isolated live and apoptosis cells. The percentage of apoptosis rate in the renal cells were analyzed based on 30,000 cells sample. Data are shown as mean ± SEM of % control from three independent experiments. $^{***}p < 0.001$ compared with control and $^{\#}p < 0.05$ compared with colistin-treated group. PC, pinocembrin.

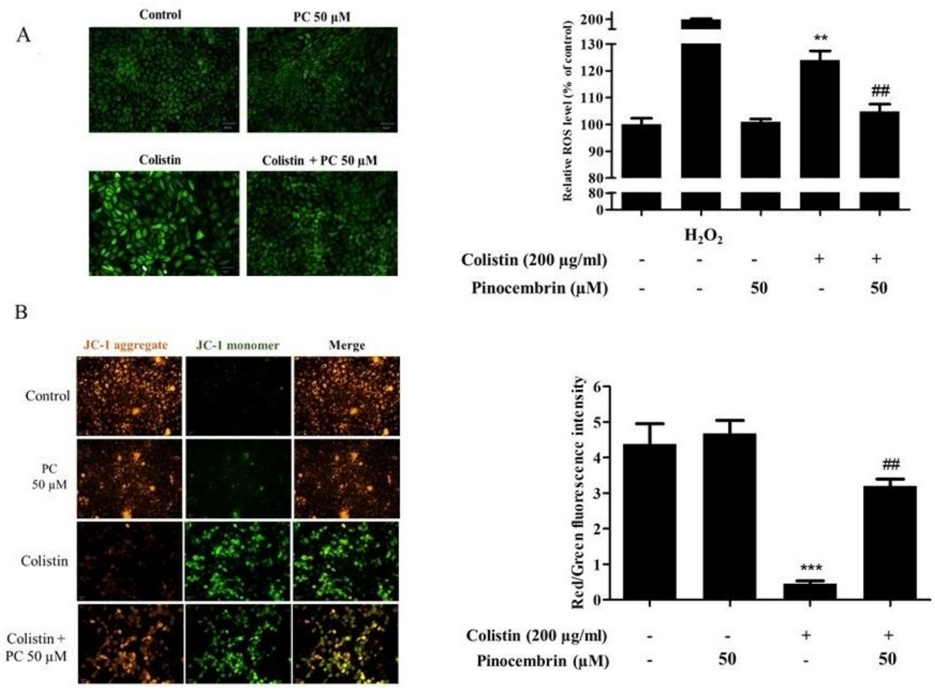

Figure 3 Protective effect of pinocembrin against colistin-induced mitochondrial damage in RPTEC/TERT1 cells. (A) The fluorescence imaging represented intracellular ROS level after colistin and/or pinocembrin treatment. Quantitative analysis of relative ROS levels normalized with control. (B) The fluorescence imaging of aggregate and monomeric forms of JC-1. Quantitative analysis of JC-1 ratio indicates membrane potential alteration. Data are shown as mean ± SEM of % control from four independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control, $^{\#\#}p < 0.01$ compared with colistin-treated group. PC, pinocembrin.

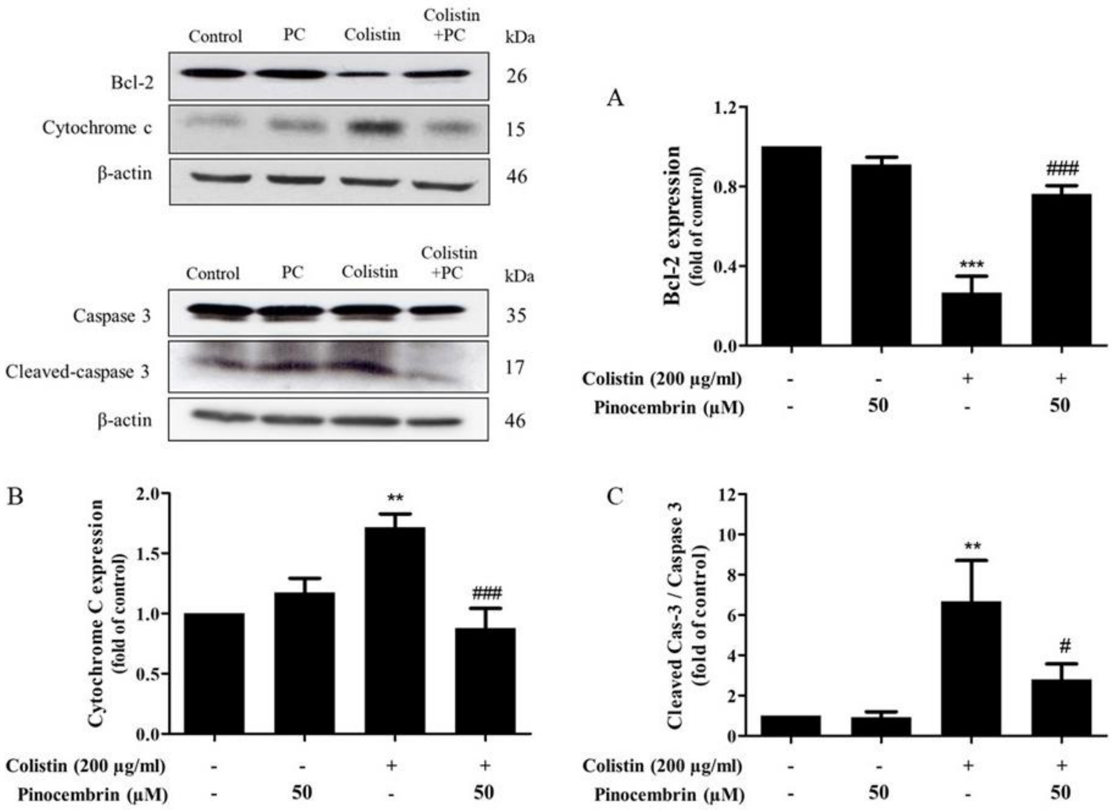

Figure 4 Protective effect of pinocembrin on protein expressions related to apoptosis pathway including (A) Bcl-2, (B) cytochrome C and (C) cleaved caspase-3 in colistin-treated RPTEC/TERT1 cells. Protein levels are normalized with β-actin showing as mean ± SEM of % control from four to five independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with colistin-treated group.

Pinocembrin partially attenuated colistin-induced apoptosis whereas it almost completely reversed the effect of colistin-induced elevation of ROS. Mitochondria impairment as analyzed by loss of MMP has been shown as a target of colistin-mediated cytotoxicity.[13] This mechanism is a downstream to colistin-mediated ROS production.[14] Our data have shown that pinocembrin protects colistin-induced loss of MMP. Cytochrome C release and caspase-3 activation are the consequence of mitochondrial damage.[15] Interestingly, we found that pinocembrin suppressed colistin-induced cytochrome C release and caspase-3 activation with their magnitude being comparable to pinocembrin's effects on colistin-induced elevation of ROS. These data indicate that targets of pinocembrin might be at ROS level induced by colistin. As ROS could occur as a result of increase generation or decrease scavenging activities[16], further studies are required to investigate the effects of pinocembrin on the mechanisms involving ROS generation and scavenging since it is beneficial for developing anti-oxidants useful for many ROS-centered diseases. In addition, this compounds significantly attenuated a decrease in expression of anti-apoptotic, Bcl-2, induced by colistin. These results imply that complex interplay between pro-apoptotic and anti-apoptotic proteins exists in response to effect of pinocembrin. Pinocembrin at 50 μM showed the maximal effect on cell viability but not completely restored cell viability. Increasing concentration to 100 μM did not produce more effect. These data indicate that pinocembrin may not directly interfere with colistin molecules. However, direct chemical interaction between colistin and pinocembrin needs further investigation to exclude potential effect of pinocembrin on therapeutic failure of colistin. The proposed mechanism by which pinocembrin reduces colistin-induced cytotoxicity of renal proximal tubular cells is shown in Fig. 5.

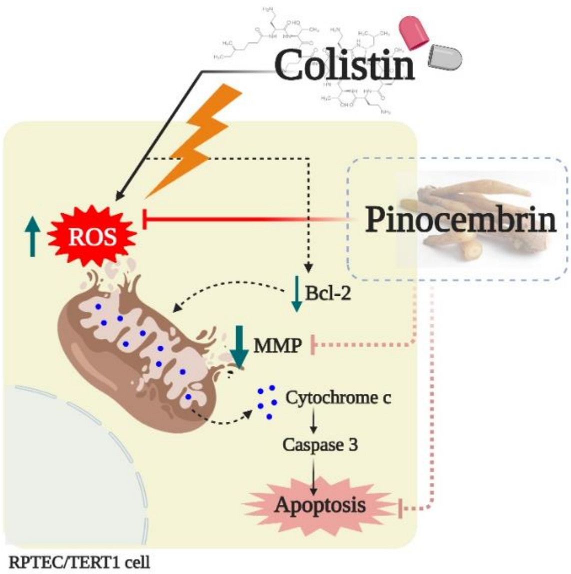

Figure 5 Proposed diagram for the protective mechanism of pinocembrin against colistin-induced human proximal tubular cell apoptosis.

Conclusion

In conclusion, this study emphasizes the actions of pinocembrin on colistin-induced apoptosis via attenuation of oxidative stress and mitochondrial impairment. Pinocembrin may exert as an agent for the prevention of nephrotoxicity induced by colistin. Efficacy investigations of pinocembrin in animals are required to support its therapeutic potential application.

Acknowledgments

This study was supported by Thailand Science Research and Innovation (grant no. RSA6280082 to S.S.) N.W. is supported by a research grant from the Development and Promotion of Science and Technology Talents Project (DPST).

Conflict of Interest

None to declare.

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