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Research Article

Pinostrobin attenuates colistin-induced apoptosis of human renal proximal tubular cells

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ABSTRACT

Colistin is one of the last-resort antibiotics used to treat multidrug-resistant (MDR) gram-negative bacterial infection. However, this drug causes nephrotoxicity by inducing oxidative stress and mitochondrial impairment of renal proximal tubular cells. Pinostrobin, which is a major natural bioactive compound isolated from *Boesenbergia rotunda*, has anti-oxidative properties and preventive effects on mitochondrial damage. Therefore, this study aimed to investigate the protective effects of pinostrobin against colistin-induced toxicity in human renal proximal tubular (RPTEC/TERT1) cells. Treatment of colistin (200 µg/ml) significantly reduced cell viability and increased apoptotic cells compared with vehicle treatment. These effects were attenuated by co-treatment with pinostrobin (50-100 µM). Colistin-induced apoptosis was correlated with increased ROS and cytochrome c expression accompanied by reduction in mitochondrial membrane potential and anti-apoptotic protein (Bcl-2) expression. These effects were abolished by co-treatment with pinostrobin. Collectively, pinostrobin has protective effects against colistin-induced apoptosis of RPTEC/TERT1 cells by improving oxidative status and mitochondrial function.

Keywords:
Colistin, Mitochondrial dysfunction, Nephrotoxicity, Pinostrobin, Renal proximal tubular cell

1. INTRODUCTION

Drug-induced nephrotoxicity, particularly in acute kidney injury (AKI), represents the most complication associated with treatment of the particular diseases. The common pathophysiology of these diseases including glomerular hemodynamic alterations, crystal formation, inflammation, and tubular cell toxicity[1-3]. An approximate 50-60% of drug-related AKI was caused by antibiotics and anti-inflammatory drugs[4,5].

Among antibiotics, colistin (or polymyxin E) represents the most important drug possessing nephrotoxicity as an adverse effect[6]. In the clinical settings, this drug is a last-line polypeptide antibiotic for the treatment of Gram-negative bacterial infection caused by MDR[7]. Moreover, colistin has been announced by World Health Organization (WHO) to use as an essential drug for the treatment of severe infections particularly the diseases caused by *Pseudomonas aeruginosa* and *Enterobacteriaceae*[8]. However, the uses of colistin are associated with AKI[9-10]. Administration of colistin causes accumulation of this drug in renal proximal tubules which further induces oxidative stress, mitochondrial damage, activation of inflammatory processes, and cell death in both *in vitro* and *in vivo* studies[9-15]. These pathologic features lead to kidney injuries as indicated by decrease in creatinine clearance level and low urine output, and kidney failure ensues. However, there are no effective therapies to diminish adverse effect of colistin. Therefore, development of novel nephroprotective agents is very challenging and important for optimizing clinical use of colistin.

Pinostrobin is a dominant natural bioflavonoid

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isolated from *Boesenbergia rotunda*, a ginger species located in Southeast Asia. This plant originally defined as a medicinal plant due to many pharmacological activities such as antioxidant against lipid peroxidation[16, 17], anti-inflammatory and anti-bacterial activities[18]. Study of Li et al., reveals that pinostrobin can suppress neurotoxin-induced neuronal cell apoptosis by altering intrinsic mitochondrial pathway[19]. Despite its prominent bioactivities, the nephroprotective effect of pinostrobin remains enigmatic. Therefore, this study aimed to investigate whether pinostrobin protects against renal cell apoptosis induced by colistin.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Pinostrobin (purity >98% by HPLC) was isolated from *Boesenbergia rotunda*. Colistin sulfate salt (15,000 units/mg), thiazolyl blue tetrazolium bromide (MTT) and trypan blue were purchased from Sigma-Aldrich (MO, USA). Apoptosis Detection Kit (Annexin V-FITC) was purchased from BD biosciences (CA, USA). Bcl-2, cytochrome c, and β-actin antibody were purchased from Cell signaling Technology (MA, USA).

2.2. Extraction and isolation of pinostrobin

The air-dried and finely powdered rhizomes of *B. rotunda* (2.5 kg) were percolated with 95% EtOH (6 L, 4 times x 7 days) at room temperature to give a crude EtOH extract (535.6 g) after solvent removal. After addition of EtOH (1 L) to the crude extract, the precipitate was filtered, washed with cold EtOH to obtain a crude solid (71.3 g) and a residue (res. 1) (460.4 g) after evaporation under reduced pressure. Recrystallization of the crude solid material from EtOH provided pure pinostrobin (26.6 g) and a residue (res. 2) (44.6 g) after evaporation under reduced pressure. The combined residue (res. 1+res. 2, 505 g) were further separated by column chromatography on silica gel, eluting with EtOAc-hexanes gradient to afford six separated subfractions (A1-A6). Subfraction A3 (123.0 g) yielded an additional amount of pure pinostrobin (41.2 g) after crystallization from EtOH. A chemical structure of pinostrobin is shown in Figure 1A.

2.3. Cell Culture

RPTEC/TERT1 cells, a human renal proximal tubular cells line, were purchased from American Type Culture Collection (VA, USA). Cells were cultured in the mixture of Dulbecco's modified Eagle's medium with Ham's F-12 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml human transferrin, 10 ng/ml recombinant human EGF, 25 ng/ml hydrocortisone, 0.05 µg/ml selenium and 5 µg/ml insulin. Cells were incubated at 37ºC in 5% CO_2 under 95% humidity. Once confluent, RPTEC/TERT1 cells were collected with 0.25% Trypsin EDTA for 20-30 minutes. Cells were grown in culture plate until 100% cell confluence before further subsequent experimental procedures. The treatment protocol has been shown in Figure 1B.

2.4. Cell viability assay

Cell viability of RPTEC/TERT1 was detected by using MTT assay and trypan blue staining method. MTT assay was performed by washed the cells twice with serum-free media and replaced with 100 µl/well of MTT solution (0.5 mg/ml) and incubated for 1 h in humidified atmosphere of 5% CO_2 at 37ºC. The supernatant was discarded, and 100 µl/well of DMSO was added to dissolve formazan crystals. The absorbance was read at 570 nm using microplate reader. For trypan blue staining, the cell suspensions were stained with 0.4% trypan blue solution in buffer isotonic salt before observed under light microscope. Unstaining cells and staining cells indicated as live cells and dead cells, respectively.

2.5. Determination of cell apoptosis

Apoptotic cells were determined by flow cytometry assay following staining with propidium iodide (PI) and an annexin V-FITC. Harvested cells were suspended with 1X biding buffer and stained with Annexin V-FITC and PI in the dark for 15 minutes. The stained cells were subjected to flow cytometry analysis (BD Biosciences, CA, USA). The results were reported as the percentage of live cells, apoptosis cells and necrosis cells in the random sampling of 30,000 cells.

2.6. Assessment of mitochondrial ROS production

The level of intracellular ROS was determined using fluorescent reagent 2',7'-dichlorofluorescin diacetate (DCFH-DA). RPTEC/TERT1 cells grown on 96-black well plate were washed with sterile DPBS and incubated with 10 µM DCFH-DA for 30 minutes at 37ºC, 5% CO_2 in the dark. After removed the dye, cells were three washed with sterile DPBS and photographed with 20X fluorescence microscope. The fluorescence absorbance was measured at excitation and emission wavelength 480 and 530 nm respectively using fluorescence plate reader (Operetta and EnVision, PerkinElmer). Relative ROS level was calculated as percentage normalized with control.

2.7. Assessment of the alteration in mitochondrial membrane potential ($\Delta\psi_m$)

The change in mitochondrial membrane potential of RPTEC/TERT1 cells was measured using JC-1 fluorescence dye. The cells on 96-back well plate were washed with DPBS and then incubated with 20 µM JC-1 reagent in DPBS for 15 min at 37ºC. Dye was removed, cells were washed again with DPBS and observed under fluorescence microscope at an emission wavelength of 488 nm. Monomeric and aggregates forms of JC-1 were emitted green and red fluorescence at 530 and 595 nm, respectively. Quantitative analysis of fluorescence intensity was measured by image J software. The ratios of red/green fluorescence intensity were interpreted as the alteration of mitochondrial membrane potential.

2.8. Assessment of apoptotic proteins expression

Total protein of RPTEC/TERT1 cells were extracted with lysis buffer (100 µl/well) and incubated on ice for 20 minutes. Whole cell suspension was centrifuged at 13,000 rpm at 4°C for 20 minutes. The supernatant was collected and stored at -80°C. An equal amount of protein samples was analyzed by Western blotting. Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked with nonfat dry milk (5%) in Tris-buffered Saline-Tween 20 (TBST) for 2 hours before overnight incubation with primary antibodies. The membranes were washed with TBST, followed by secondary antibody (horseradish peroxidase-conjugated) incubation for 1 hour. Finally, the immune complexes were detected using Electro-Chemi-Luminescence (ECL) and exposed to UltraCruz autoradiography films (Santa Cruz, CA). The protein bands intensity was analyzed and quantified using Image J software.

2.9. Data analysis and statistical methods

All data are expressed as means±SEM. Figures were prepared using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The statistical significance between control and treatment groups was analyzed using one-way ANOVA followed by Tukey test for multigroup comparisons. A *P* value <0.05 was considered significant difference.

3. RESULTS

3.1. Pinostrobin attenuates colistin-induced toxicity in renal proximal tubular cells

The protective effects of pinostrobin on colistin-induced cellular toxicity were determined using MTT and trypan blue staining assays in RPTEC/TERT1 cells. As shown in Figure 2A, cell viability was reduced by increasing concentration of colistin with the IC_{50}~200 µg/mL at 72 h. Interestingly, this cytotoxicity was attenuated by co-treatment with pinostrobin in a concentration-dependent manner with maximal effect being observed at 50 µM (Figure 2B). Similarly, the results were consistent with trypan blue staining method (Figure 2C). These results suggest protective effects of pinostrobin on colistin-induced proximal tubular cytotoxicity.

3.2. Pinostrobin alleviates colistin-induced apoptostic cells death

Colistin causes nephrotoxicity by induction of apoptosis. To determine whether pinostrobin protected proximal tubular cells against colistin-induced apoptosis,

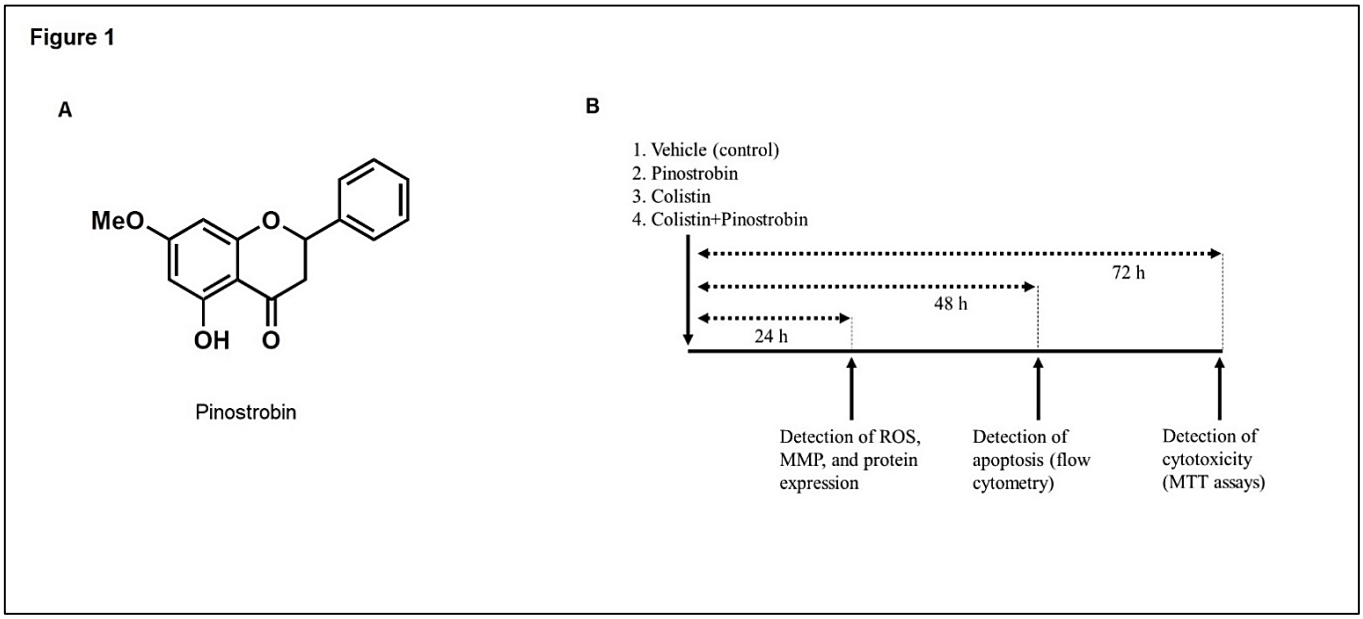

Figure 1. Chemical structure of pinostrobin (A) and experimental protocol (B).

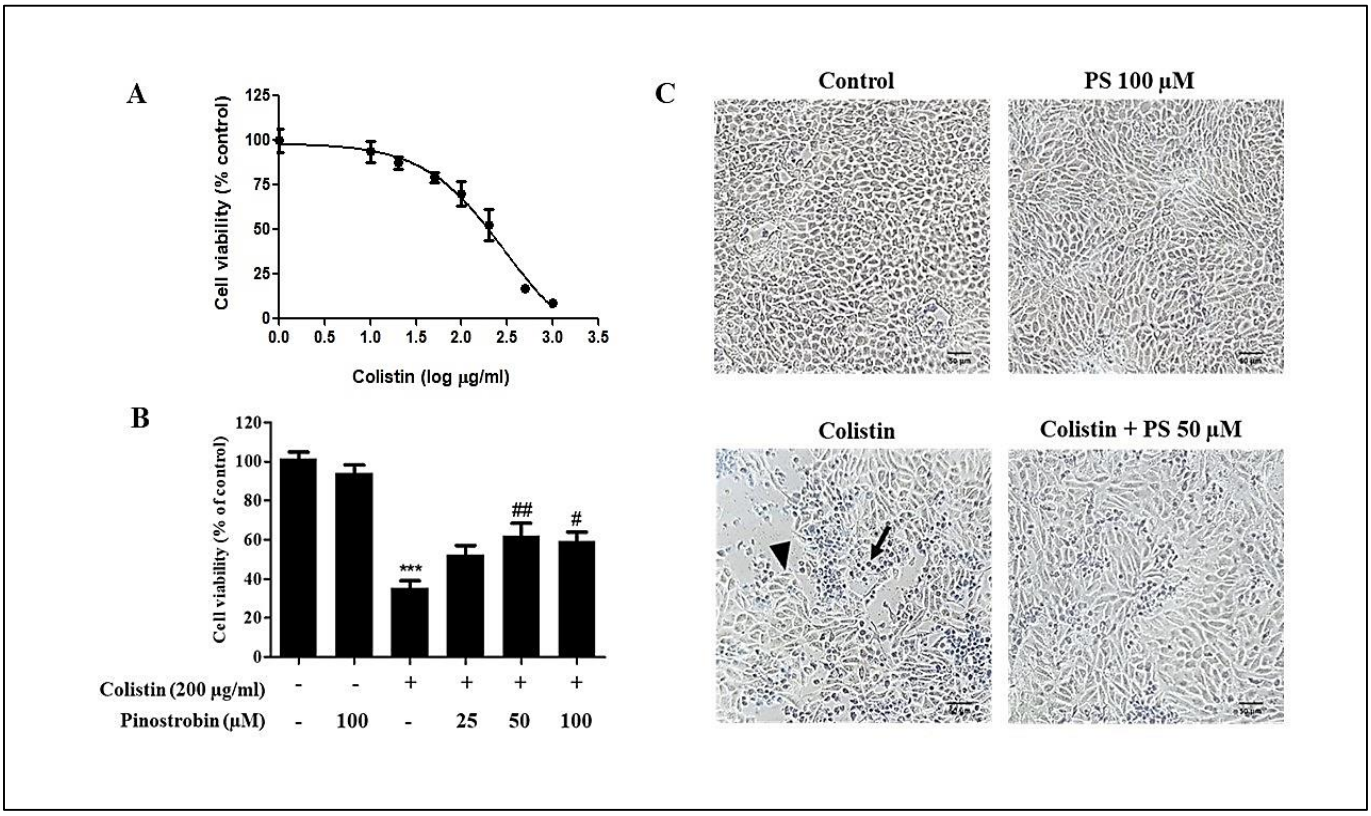

Figure 2. Effect of pinostrobin on cell viability after colistin exposure. (A) Concentration-response relationship of colistin on cytotoxicity at 72 h treatment as analyzed by MTT assays. (B) The viability of RPTEC/TERT1 cells after 72 hours treated with vehicle, 100 µM pinostrobin, 200 µg/ml colistin, and 200 µg/colistin plus 25, 50 or 100 µM pinostrobin. (C) Imaging of cells stained with trypan blue indicated dead cell (arrow) and live cell (triangle). The data are represented as mean±SEM of % control; n=3. $^{*}P<0.001$ compared with control, $^{\#}P<0.05$ compared with colistin-treated group. PS represents pinostrobin.

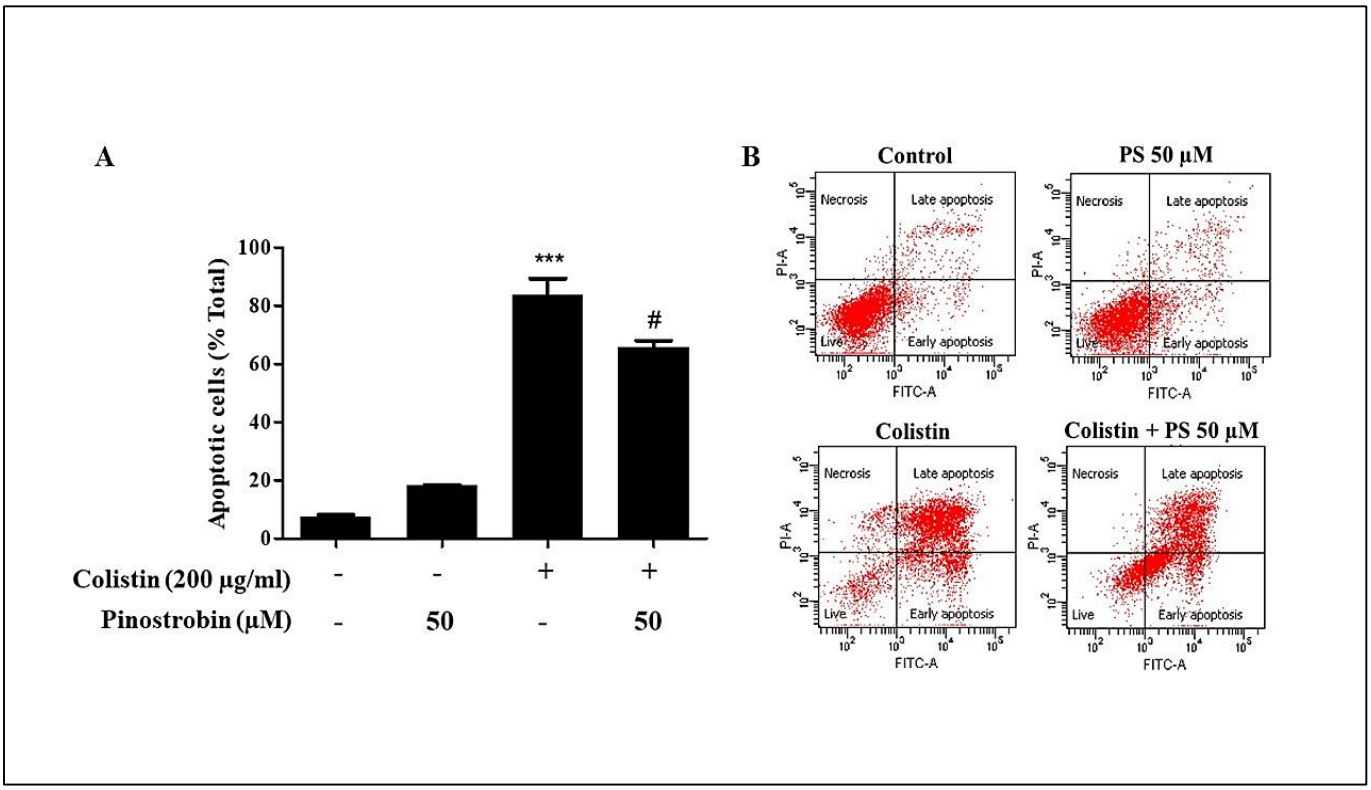

Figure 3. Effect of pinostrobin on apoptosis of RPTEC/TERT1 cells. Apoptotic cells were analyzed by flow cytometry after 48 hours of treatment with vehicle, 50 µM pinostrobin, 200 µg/ml colistin and 200 µg/ml colistin plus 50 µM pinostrobin. Data are shown as mean±SEM of % control: n=3. $^{*}P<0.001$ compared with control, $^{\#}P<0.05$ compared with colistin-treated group. PS represents pinostrobin.

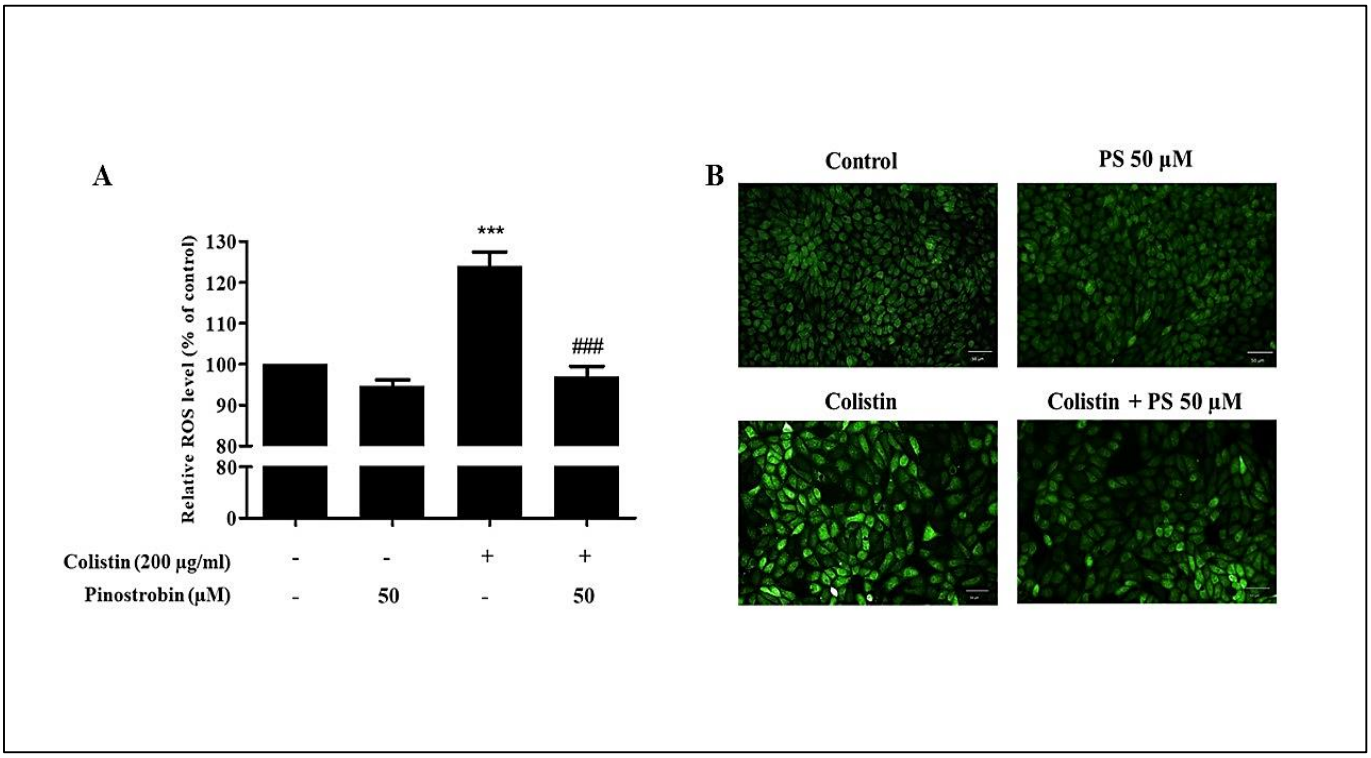

Figure 4. Effect of pinostrobin on colistin-stimulated ROS production in RPTEC/TERT1 cells. (A) Fluorescence imaging of ROS production in renal proximal tubular cells after 24 h incubated with vehicle, 200 µg/ml colistin and 200 µg/ml colistin plus 50 µM pinostrobin. (B) Relative ROS levels for each condition compared with control. The data are represented as mean±SEM of % control form 4 experiments. $^{*}P$<0.001 compared with control, $^{\#}P$<0.001 compared with colistin-treated group. PS represents pinostrobin.

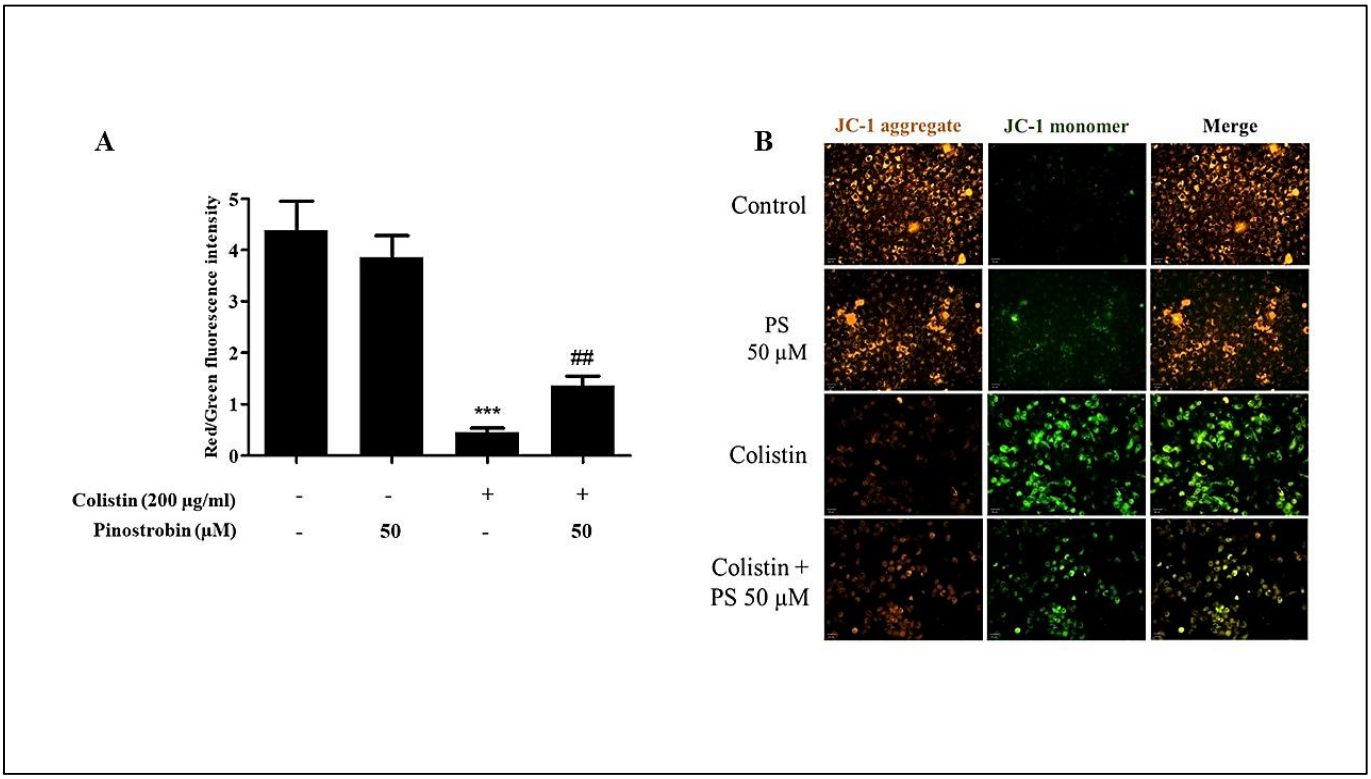

Figure 5. Effect of pinostrobin on colistin-induced reduction of mitochondrial membrane potential (MMP; ψ_m). Alteration of mitochondrial membrane potential detected by JC-1 staining of RPTEC/TERT1 cells after 24 h treated with 200 µg/ml colistin and 200 µg/ml colistin plus 50 µM pinostrobin. (A) Fluorescence imaging of JC-1 aggregate form (represented normal MMP function; red color) and JC-1 monomeric form (represented damaged MMP; green color). (B) Quantitative analysis indicated the ratio between red and green fluorescence intensity. Decrease in the ratio was clarified as loss of ψ_m. The data are represented as mean±SEM of % control from n = 3-4. $^{*}P$<0.001 compared with control, $^{\#}P$<0.01 compared with colistin-treated group. PS represents pinostrobin.

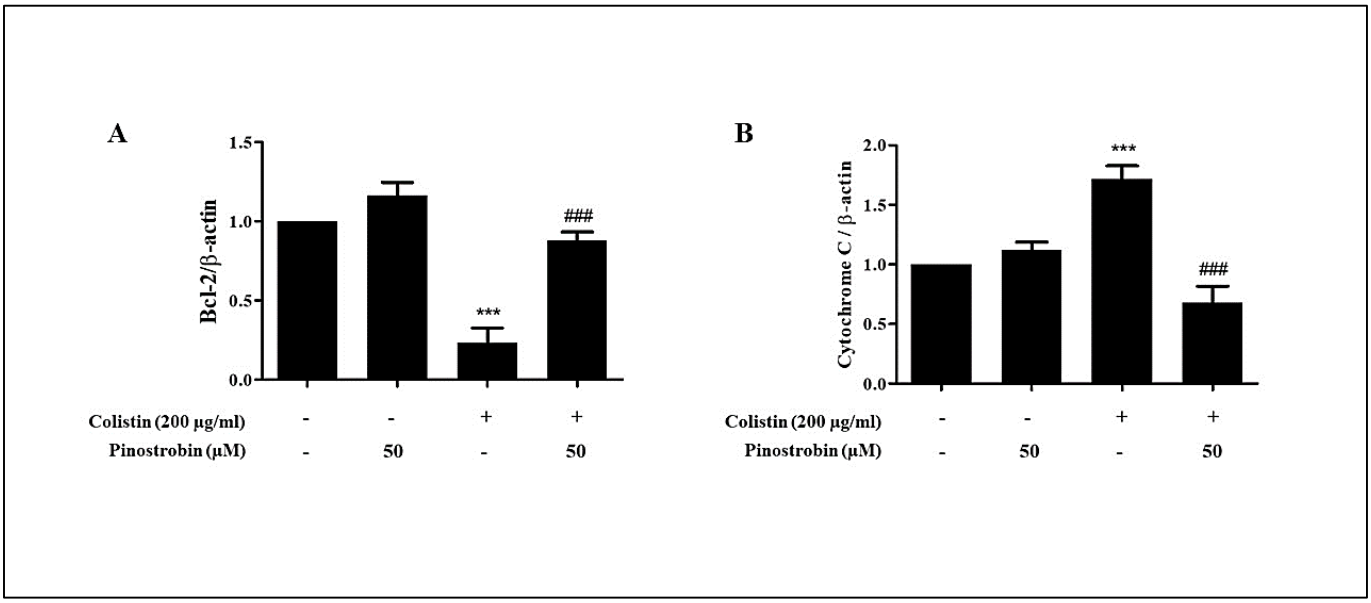

Figure 6. Effect of pinostrobin on expression of apoptosis proteins in RPTEC/TERT1 cells. Protein expressions of Bcl-2 (A) and cytochrome c (B) normalized with β-actin are shown. The cells were treated with colistin with or without pinostrobin (50 µM) for 24 h before protein extraction. The results are represented as mean±SEM of % control obtained from 3 experiments. $^{*}P<0.001$ compared with control, $^{\#}P<0.001$ compared with colistin-treated group. PS represents pinostrobin.

the percentage of cell death was analyzed by flow cytometry using annexin V-FITC/PI staining assay in RPTEC/TERT1 cells. Treatment of colistin (200 µg/ml) for 48 h significantly augmented early and late apoptosis compared with the control (Figure 3). As expected, co-treatment of pinostrobin (50 µM) significantly attenuated apoptosis induced by colistin. Thus, these data suggest that pinostrobin can reduce colistin-induced cell apoptosis.

3.3. Colistin-induced mitochondrial dysfunction is ameliorated by pinostrobin in renal proximal tubular cells

Colistin induces cellular toxicity via stimulation of ROS production[14]. To examine whether pinostrobin reduced colistin-induced ROS levels, the effect of pinostrobin on ROS levels induced by colistin was determined using DCFH-DA assays. Incubating RPTEC/TERT1 cells with colistin significantly promoted higher cellular level of ROS compared with control cells. The ROS was significantly reduced when co-treated the cells with colistin and with 50 µM pinostrobin (Figure 4). This data indicates that ROS-activated by colistin can be suppressed by pinostrobin. Next, the effect of pinostrobin on colistin-induced mitochondrial damage in RPTEC/TERT1 cells was determined. As shown in Figure 5, colistin significantly decreased the ratio of red/green fluorescence intensity indicative of reduction in mitochondrial membrane potential (ψ_m). Co-treatment of colistin and pinostrobin significantly increased the ratio of red/green fluorescence intensity indicating that pinostrobin restore in mitochondrial membrane potential in RPTEC/TERT1 cells.

3.4. Pinostrobin reduces cytochrome C and up-regulates anti-apoptotic Bcl-2 proteins expression

Colistin induces cell apoptosis by releasing pro-apoptotic cytochrome c proteins from mitochondria to cytosol and reducing anti-apoptotic Bcl-2 protein expression[9]. Therefore, we explored the mechanism of pinostrobin on colistin-induced apoptosis in RPTEC/TERT1 cells. Colistin significantly increased expression of cytochrome c, accompanied by reduction in Bcl-2 expression (Figure 6A and Figure 6B). As expected, co-treatment the cells with colistin and pinostrobin (50 µM) reversed the expression of cytochrome c and Bcl-2. Of note, treatment of pinostrobin alone had virtually no effect on cytochrome c and Bcl-2 expression. These data suggest that apoptotic proteins regulated by colistin were restored by pinostrobin.

4. DISCUSSION

Even though the use of colistin was limited due to the side effect associated with nephrotoxicity, this drug is still the last-line therapeutic option to treat MDR bacterial infection. Thus, development of nephroprotective agents to ameliorate renal injury is urgently needed. Previously, some therapeutic strategies including ascorbic acid[20], baicalein[21] or lycopene[22] were introduced to relieve the undesirable effects of colistin. Whereas most of them showed protective effect of renal cells against colistin-induced toxicity in the *in vitro* and *in vivo* studies, none of the studies reported clinical utility

of that compounds. Therefore, finding the novel natural products is important for identification of drug candidate beneficial for further drug development. In the present study, we revealed for the first time that pinostrobin protected renal cell toxicity induced by colistin. Pinostrobin is a chiral flavonoid found in *B. rotunda* harboring various pharmacological activities[16-18]. Our results are based on an immortalized epithelial cell line derived from human renal proximal tubule (RPTEC/TERT1 cells) which express several key transporters useful for determination of xenobiotic transports[23].

According to our preliminary results, colistin induced renal cell toxicity in concentration- and time-dependent manner as analyzed by MTT assays in RPTEC/TERT1 cells. Colistin treatment at 200 µg/ml for 72 hours represents the most appropriate condition causing toxicity based on IC_{50} value. Amelioration of the cytotoxic effect of colistin was observed in the presence of pinostrobin. Concentration-response relationship demonstrated that maximal protective effect of pinostrobin on colistin-induced toxicity achieved at 50 µM with no further response at higher concentration. Therefore, pinostrobin at 50 µM was selected for further experiments. The protective effects of this compound were confirmed using flow cytometry. we observed that pinostrobin at 50 µM reduced colistin-induced apoptosis with the similar level as noticed in MTT assays, suggesting that anti-apoptotic effects of pinostrobin completely explain the protective effects of pinostrobin. As mentioned in other studies, colistin-induced tubular cell apoptosis is mediated by oxidative stress production and mitochondrial damage[14,15]. Our data show that pinostrobin completely attenuated colistin-induced ROS level with the degree being higher than its anti-apoptotic effects. Therefore, reduction of ROS might not explain all anti-apoptotic mechanisms of pinostrobin. Of note, it is interesting to investigate the anti-oxidative mechanisms of pinostrobin which might be inhibition of ROS production or stimulation of ROS destruction. In addition, opening of mitochondrial permeability transition pore (mPTP) decreases mitochondrial membrane potential (MMP; $\Delta\psi_m$) leading to mitochondrial dysfunction[24]. Our present study demonstrates that co-treatment with pinostrobin significantly attenuated the loss of MMP-induced by colistin which might be a partial mechanism of anti-apoptotic effects of pinostrobin.

Release of cytochrome c, a key protein regulating apoptosis cascade, from mitochondria is a consequence after MMP disruption[25]. In addition, pinostrobin prevented mitochondria damage induced by colistin supported by a decrease in cytochrome c protein expression following pinostrobin treatment. Upon the colistin treatment, pinostrobin not only suppressed the induction of pro-apoptotic cytochrome c protein level, but also restored an anti-apoptotic protein, Bcl-2. Taken together, the present study reveals the preventive effect of pinostrobin on colistin-induced renal proximal tubular cell apoptosis. Although pinostrobin shows a potential agent for preventing of colistin-induced renal toxicity, the information concerning the pharmacological effects of pinostrobin needs further investigation in animal and human studies.

5. CONCLUSIONS

This study has demonstrated that pinostrobin shows a protective effect against colistin-induced apoptosis of human renal proximal tubular cells. The protective effect of pinostrobin is mediated by a decrease in oxidative stress and prevention of mitochondrial damage.

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Conflict of interest
The authors declare no conflict of interest.

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Ethics approval
None to declare.

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