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ORIGINAL ARTICLE

Ameliorative effect of taxifolin on gentamicin-induced ototoxicity via down-regulation of apoptotic pathways in mouse cochlear UB/OC-2 cells

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Abstract

Background: Taxifolin is a flavanonol with efficacious cytoprotective properties, such as anti-inflammatory, antioxidant, anticancer, hepatoprotective, and nephroprotective effects. However, the potential protective effects of taxifolin against gentamicin-induced ototoxicity have not been confirmed. In this study, the possible mechanisms underlying the effects of taxifolin on gentamicin-induced death of UB/OC-2 cochlear cells were investigated.

Methods: Mouse cochlear UB/OC-2 cells with or without taxifolin pretreatment were exposed to gentamicin, and the effects on cytotoxicity, reactive oxygen species (ROS) production, mitochondrial permeability transition, and apoptotic marker expression were examined using biochemical techniques, flow cytometry, western blotting, and fluorescent staining.

Results: Little or no apparent effect of taxifolin on cell viability was observed at concentrations less than 40 μM. Further investigations showed that gentamicin significantly inhibited cell viability in a concentration-dependent manner. Pretreatment with taxifolin attenuated gentamicin-induced lactate dehydrogenase release, as well as cellular cytotoxicity. In addition, taxifolin significantly prevented gentamicin-induced cell damage by decreasing ROS production, stabilizing mitochondrial membrane potential, and downregulating the mitochondrial pathway of apoptosis.

Conclusion: In summary, pretreatment with taxifolin is effective for mitigating gentamicin-induced apoptotic cell death mediated by the mitochondrial pathway. Our data suggest that taxifolin provides a new approach to combat gentamicin-induced ototoxicity.

Keywords: Apoptosis; Gentamicin; Ototoxicity; Reactive oxygen species; Taxifolin

1. INTRODUCTION

Aminoglycoside antibiotics are commonly used in the treatment of bacterial infections; they work by disrupting the integrity of the bacterial cell membrane, which causes pore formation and finally leads to cell death.[1] Gentamicin, an aminoglycoside antibiotic, is used to treat many gram-negative and some gram-positive infections. Gentamicin kills bacteria by binding to the bacterial 30S ribosomal subunits, thereby inhibiting protein synthesis, and is widely used because of its low price, low rate of resistance, and excellent activity.[2,3] However, the clinical utility of gentamicin is limited by its reversible nephrotoxicity and irreversible ototoxicity.[4–8] Patients receiving gentamicin treatment can experience hearing loss or deranged vestibular function.[9] Ototoxic hearing loss has long-term individual and societal costs.[10]

The abnormal accumulation of reactive oxygen species (ROS) plays an integral role in aminoglycoside-induced ototoxicity and is thought to result in the death of cochlear hair cells.[11–13] Noticeable protective effects have been reported for numerous antioxidant agents, such as N-acetylcysteine, D-methionine, resveratrol, and Coenzyme Q10.[14–17] A combination of resveratrol and N-acetylcysteine reduces aminoglycoside ototoxicity in response to oxidative stress and inflammation-related genes.[18] Dihydromyricetin, a flavonoid found in rattan tea, inhibits gentamicin-induced ototoxicity by suppressing ROS production.[19] These findings suggest that compounds with antioxidant effects can ameliorate or prevent gentamicin damage in cochlear hair cells.

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Gentamicin leads to cellular damage by modifying a number of cellular components, including DNA base oxidation, protein carbonylation, and lipid peroxidation.[20] Gentamicin-induced ototoxicity is associated with excessive ROS production and subsequent apoptosis.[21] A previous study demonstrated that ROS affect mitochondrial permeability transition pore opening and trigger the apoptotic pathway through mitochondrial cytochrome *c*-mediated caspase activation.[22,23] Cytochrome *c* is released from the mitochondria to the cytoplasm, which then induces the activation of Caspase-9, Caspase-3, and poly(ADP-ribose) polymerase (PARP) as cleaved forms, leading to failed DNA break repair and apoptotic cell death.[23]

Taxifolin, widely distributed in vegetables and plants, has potential as a functional food ingredient because of its health-promoting effects, including antioxidant, anticancer, and anti-inflammatory properties.[24,25] Taxifolin is an effective antioxidant because of the presence of five phenolic hydroxyl groups in its structure.[26] Recent studies have reported that taxifolin provides hepatoprotective activity through the MAPK/Nrf2 antioxidant pathway.[27] Taxifolin has been shown to possess anticancer properties by diminishing ZEB signaling through AKT.[28] It has also been shown that taxifolin treatment can significantly attenuate rotenone-induced renal dysfunction.[29]

The combination of different drugs could reduce ototoxic side effects without the cost of developing a new antibiotic or compromising antimicrobial activity.[30] Several reports have suggested that antioxidant compounds could potentially diminish the ototoxicity of aminoglycoside antibiotics.[27,29] However, the potential protective effect of taxifolin against aminoglycoside antibiotic-induced ototoxicity has not been confirmed. The purpose of this study was to investigate the protective effect of taxifolin on gentamicin-induced ototoxicity and its underlying mechanisms.

2.METHODS

2.1. Sample extraction

Polygoni orientalis Fructus (1 kg) was placed in 55% ethanol and heated to the boiling point under reflux for 2 hours. The ethanol extract was concentrated by evaporation using a rotary evaporator at 60°C to remove excess ethanol and then triturated with n-hexane, dichloromethane, ethyl acetate, and n-butanol. The ethyl acetate-soluble fraction was purified using column chromatography. Taxifolin quantification and identification were carried out using high-performance liquid chromatography.

2.2. Cell culture

The immortalized mouse cochlea cell line UB/OC-2 was obtained from Ximbo (London, United Kingdom) and cultured at 33°C in a humidified atmosphere of 5% CO_2 in MEM supplemented with GlutaMAX (Gibco, NY, USA), 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA), and 50 U/mL of IFN-γ (R&D Systems, Minneapolis, MN, USA).

2.3. Cell viability test

Cell viability was determined through colorimetric measurements using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; VWR International, Radnor, PA, USA) solution. Cells (5×10^4 cells per well) were seeded in 24-well plates and incubated overnight under permissive conditions. After treatment, 0.2 mg/mL MTT solution was added for 4 hours. The formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm using a microplate reader (Infinite 200 PRO Series Multimode Reader; TECAN, Switzerland).

2.4. Morphological observation

The cells were pretreated with 10, 20, and 40 μM taxifolin for 2 hours and co-incubated with 1.25 mM gentamicin for 24 hours. After treatment, the changes in cell morphology were captured under 200× magnification using an Olympus BX41 microscope (Tokyo, Japan).

2.5. Cell cytotoxicity test

Cell cytotoxicity was determined using a colorimetric assay based on lactate dehydrogenase (LDH) release. The cells were pretreated with 10, 20, and 40 μM taxifolin for 2 hours and co-incubated with 1.25 mM gentamicin for 24 hours. After treatment, cell cytotoxicity was assessed using the LDH Cytotoxicity Assay Kit (Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's protocol.

2.6. Cellular ROS assay

The cellular levels of ROS were detected using the fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFDA; Enzo Life Sciences). The cells were pretreated with 10, 20, and 40 μM taxifolin for 2 hours and co-incubated with 1.25 mM gentamicin for 24 h. After treatment, the cells were collected and stained using DCFDA dye (Enzo Life Sciences). The cells were then washed with ice-cold phosphate-buffered saline (PBS) and incubated with 10 μM DCFDA dye for 30 minutes. The cells were collected, and fluorescence intensity was analyzed using a BD Accuri C6 flow cytometry system (BD Biosciences, San Jose, CA, USA).

2.7. Mitochondrial membrane potential assay

Mitochondrial membrane potential was monitored using the fluorescent dye JC-1 (Enzo Life Sciences). The cells were pretreated with 10, 20, and 40 μM taxifolin for 2 hours and co-incubated with 1.25 mM gentamicin for 24 hours. After treatment, the cells were collected and stained using JC-1 dye (Enzo Life Sciences). Then, cells were washed with ice-cold PBS and incubated with 5 μg/mL JC-1 dye for 10 minutes. The cells were finally collected, and fluorescence intensity was measured using a BD Accuri C6 flow cytometry system.

2.8. Western blot analysis

The cells were pretreated with 10, 20, and 40 μM taxifolin for 2 hours and co-incubated with 1.25 mM gentamicin for 48 hours. After treatment, the cells were collected and lysed with lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). Proteins of cellular lysates were resolved by 8% to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA), which had been blocked with 3% bovine serum albumin for 1 hour. The membranes were probed with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Primary antibodies against Bcl-2, Bax, cytochrome *c*, COX IV, β-actin, Caspase-9, cleaved Caspase-9, Caspase-3, cleaved Caspase-3, PARP, and cleaved PARP were obtained from Cell Signaling Technology (Beverly, MA, USA). Goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody were purchased from PerkinElmer Life Sciences (Boston, MA, USA). The protein signals were visualized with enhanced chemiluminescence (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and detected using the KETA C Chemi imaging system (Wealtec Corporation, Sparks, NV, USA).

2.9. Subcellular fractionation

Cytosolic and mitochondrial fractions were performed according to the manufacturer's instructions (Abcam, Cambridge, MA, USA). The cells were pretreated with 10, 20, and 40 μM taxifolin

for 2 hours and co-incubated with 1.25 mM gentamicin for 48 hours. After treatment, the cell pellet was collected and resuspended in 200 μL of Extraction Buffer A (0.015% EDTA and 0.36% Tris). Then, we added the same amount of Extraction Buffer B (0.015% EDTA, 0.36% Tris, and 0.001% digitonin) and incubated samples for 7 minutes at room temperature. The mixture was centrifuged for 5 minutes at 10 000*g*, and the supernatant was immediately transferred to new tubes (cytosolic fraction). The insoluble fraction was resuspended in 200 μL of Extraction Buffer A and the same amount of Extraction Buffer C (0.03% EDTA and 0.75% Tris) for 10 min at room temperature. The mixture was then centrifuged for 5 minutes at 10 000*g*, and the supernatant was immediately transferred to new tubes (mitochondrial fraction). Finally, cytosolic and mitochondrial fractions were stored at −80°C for future analysis.

2.10. Apoptosis assay

The percentage of apoptotic cells was analyzed by flow cytometry using annexin V-FITC and propidium iodide (PI) double staining. The cells were pretreated with 10, 20, and 40 μM taxifolin for 2 hours and co-incubated with 1.25 mM gentamicin for 48 hours. After treatment, the cells were stained using the Annexin V-FITC Apoptosis Detection Kit (Abcam) following the manufacturer's protocol. The cells were resuspended in 400 μL of 1× binding buffer and stained with Annexin V-FITC and PI for 15 minutes. The ratio of apoptotic cells was analyzed using the BD Accuri C6 flow cytometry system.

2.11. Nucleic acid staining

Apoptotic chromatin changes were evaluated by DNA staining using Hoechst 33258 dye (Enzo Life Sciences), which produces bright blue fluorescence upon binding. The cells were pretreated with 10, 20, and 40 μM taxifolin for 2 hours and co-incubated with 1.25 mM gentamicin for 24 hours. After treatment, the cells were stained with Hoechst 33258 dye. Cells were washed with ice-cold PBS and fixed with methanol at −20°C overnight. The fixed cells were washed with PBS and incubated with 20 μg/mL Hoechst 33258 for 20 minutes. Fluorescence intensity was measured at an excitation wavelength of 352 nm using an Olympus BX41 microscope.

2.12. Statistical analysis

Data are presented as the mean ± SD. Student's *t*-tests were used to compare the two groups. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Effects of taxifolin and gentamicin on cell viability

Initially, we assessed the cell viability profile of taxifolin-treated UB/OC-2 cells using the MTT assay. The cells were stimulated with taxifolin at concentrations of 10, 20, and 40 μM for 48 hours. Taxifolin treatment alone had little or no effect on cell viability (Fig. 1A). Next, we chose the optimum concentration of gentamicin to induce UB/OC-2 cell damage. As shown in Fig. 1B, cell viability decreased as gentamicin increased in a significant, concentration-dependent manner at concentrations of 0.75, 1, 1.25, and 1.5 mM. At 1.25 mM gentamicin, 54% viable cells were observed. Based on these results, we used gentamicin at a 1.25 mM concentration in the following experiments.

3.2. Effects of taxifolin on gentamicin-induced ototoxicity

To investigate the effects of taxifolin on gentamicin-treated UB/OC-2 cells, we first tested the changes in cell morphology. As shown in Fig. 2A, the morphological changes, including UB/OC-2 cell shrinkage and membrane fragmentation caused by gentamicin, were reversed by taxifolin. To evaluate the otoprotective effect of taxifolin on UB/OC-2 cells, cells were treated with gentamicin and cytotoxicity was measured using an LDH assay. As shown in Fig. 2B, compared to that in the control group, the release of LDH by UB/OC-2 cells was elevated from 2.28 ± 1.63 % to 32.35 ± 2.12% after stimulation with gentamicin but decreased to 24.44 ± 4.85, 20.61 ± 3.29, and 15.30 ± 1.73% after the introduction of 10, 20, and 40 μM taxifolin for 24 hours, respectively. These data indicate that taxifolin might provide effective protection against gentamicin-induced ototoxicity.

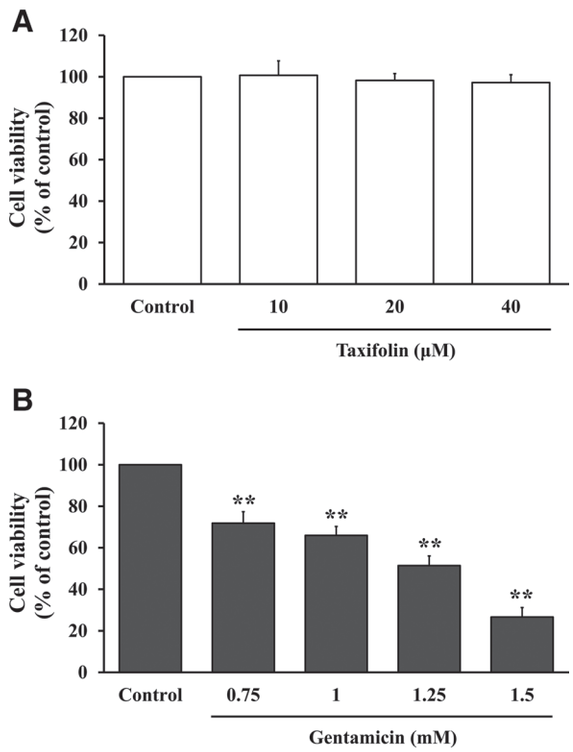

Fig. 1 Effects of taxifolin and gentamicin on viability of UB/OC-2 cochlear cells. The cells were cultured with (A) 10–40 μM taxifolin and (B) 0.75–1.5 mM gentamicin for 48 h, and cell viability was analyzed using MTT assay. The control group is considered as 100% viability. **$p < 0.01$ compared with the control group.

3.3. Taxifolin reduces gentamicin-induced intracellular ROS production and reverses mitochondrial membrane potential loss

A previous study reported that the overproduction of intracellular ROS causes a loss of mitochondrial membrane potential and triggers the intrinsic apoptotic mechanism.[31] To investigate the effect of taxifolin on oxidative damage in UB/OC-2 cells induced by gentamicin, cells were pretreated with taxifolin at concentrations of 10, 20, and 40 μM for 2 h and co-incubated with 1.25 mM gentamicin for 24 hours. Flow cytometry was used to measure intracellular ROS production using the fluorescence dye DCFDA. As shown in Fig. 3A, DCFDA-positive signals were significantly increased after stimulation with gentamicin, but these were decreased by the introduction of taxifolin in a concentration-dependent manner. No significant effect on intracellular ROS generation was observed after treatment with 40 μM taxifolin alone. Notably, taxifolin might alleviate gentamicin-induced ROS generation.

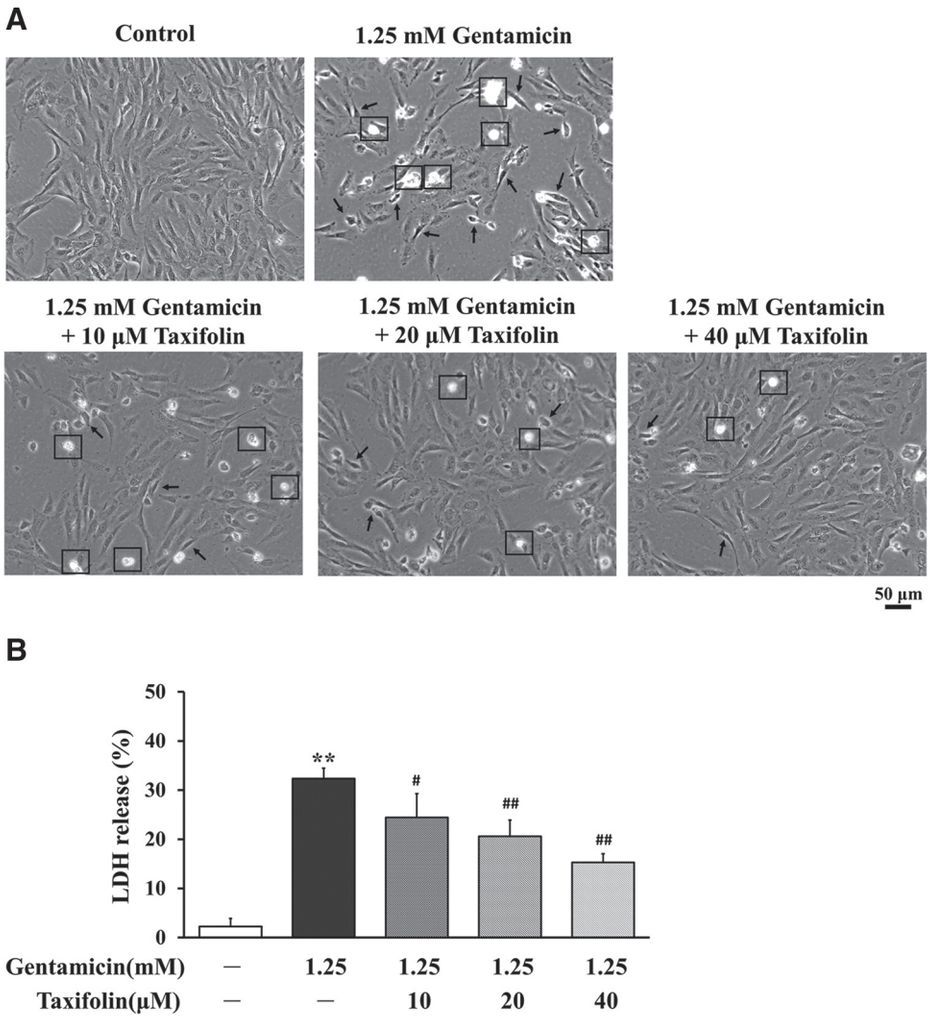

Fig. 2 Effects of taxifolin on gentamicin-induced ototoxicity. The cells were pretreated with 10, 20, and 40 μM of taxifolin for 2 h and then co-incubated with 1.25 mM gentamicin for 24 h. (A) Cellular morphology was detected using a visible light microscopy under 200× magnification. Arrows indicate morphological changes with cell shrinkage, and squares describe morphological changes with membrane fragmentation. Scale bar = 50 μm. (B) Cell cytotoxicity was assayed by measuring LDH release. $^{**}p < 0.01$ compared with the control group; $\#p < 0.05$, $\#\#p < 0.01$ compared with the gentamicin-treated group. LDH = lactate dehydrogenase.

Next, the mitochondrial membrane potential was determined by flow cytometry using the JC-1 fluorescence dye, which exhibits a green signal as the mitochondrial membrane depolarizes. The ratio of green/red fluorescence in the gentamicin-treated group increased by 3.4-fold compared with that in the control group. However, the taxifolin-treated groups had a lower green/red fluorescence ratio than the gentamicin-treated group (Fig. 3B). These data suggested that taxifolin protected UB/OC-2 cells from gentamicin-induced oxidative toxicity.

3.4. Taxifolin modulates gentamicin-induced apoptosis-related proteins

ROS production induced by gentamicin is associated with mitochondrial dysfunction, which can lead to apoptotic cell death via the loss of mitochondrial membrane potential.[22] Recent studies have revealed that the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 are involved in the regulation of mitochondrial pathway apoptosis.[32] To examine the effect of taxifolin on the apoptosis pathway induced by gentamicin, the expression of Bax and Bcl-2 was evaluated. As shown in Fig. 4A, taxifolin treatment enhanced the expression of Bcl-2 and reduced that of Bax. Our observations revealed that treatment with gentamicin increased the release of cytochrome *c* from the mitochondrial fraction to the cytosolic fraction, but cytochrome *c* was decreased in the cytosolic fraction and increased in the mitochondrial fraction in the taxifolin-treated groups (Fig. 4B). Next, we evaluated the relative expression of apoptosis-related proteins by western blot analysis. The expression of cleaved Caspase-9, cleaved Caspase-3, and cleaved PARP was significantly increased by exposure to gentamicin but decreased by the introduction of taxifolin (Fig. 5). These results suggest that taxifolin moderates gentamicin-induced apoptosis in UB/OC-2 cochlear cells.

3.5. Taxifolin attenuates apoptosis and nuclear condensation in gentamicin-treated UB/OC-2 cells

To examine whether gentamicin is involved in apoptotic cell death and whether taxifolin can attenuate gentamicin-induced apoptosis, we analyzed apoptosis using Annexin V staining and the chromatin-specific dye Hoechst 33258. Results showed

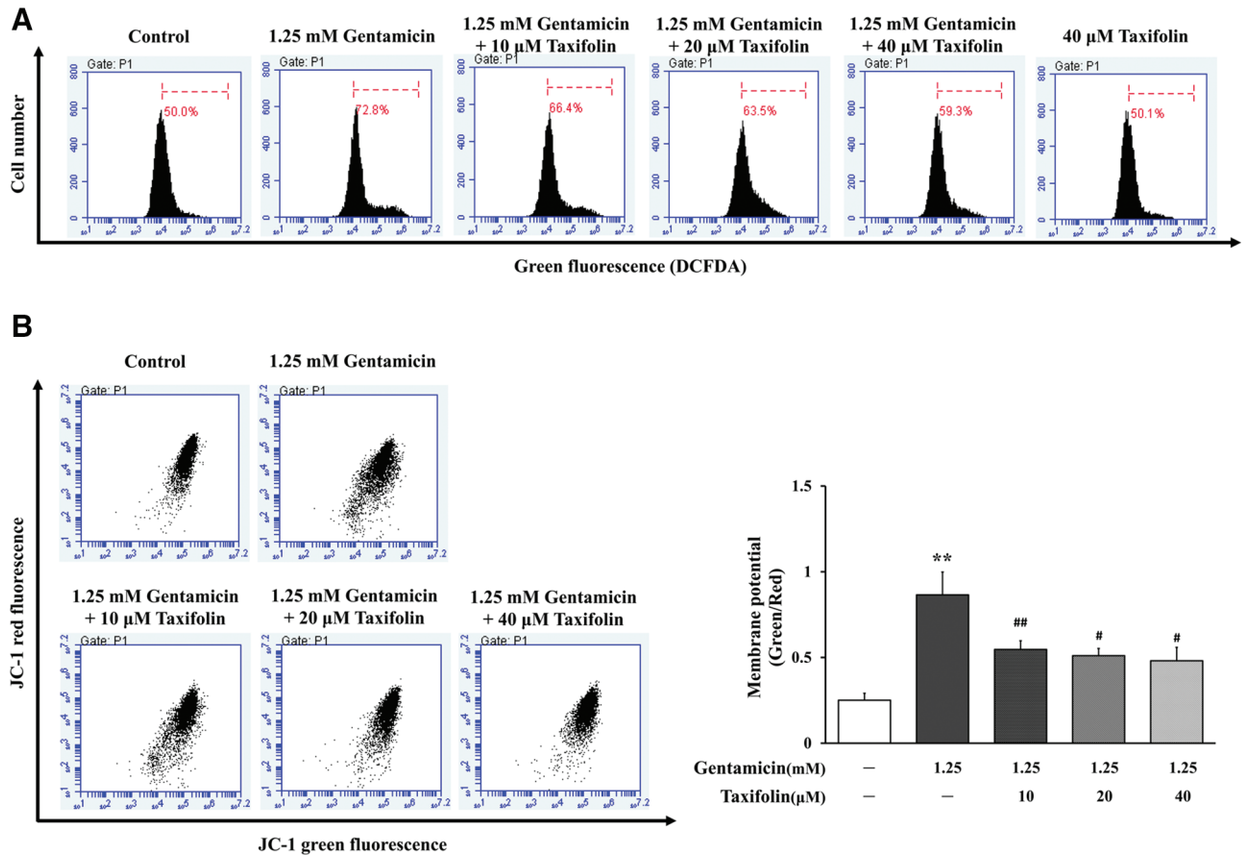

Fig. 3 Effects of taxifolin on gentamicin-induced reactive oxygen species (ROS) production and mitochondrial membrane potential changes. A, UB/OC-2 cochlear cells were pretreated with 10, 20, and 40 μM taxifolin for 2 h and co-incubated with 1.25 mM gentamicin for 24 h or 40 μM taxifolin for 24 h. ROS generation was analyzed using DCFDA dye. The fluorescence was monitored using flow cytometry. B, Changes in mitochondrial membrane potential were detected using JC-1 dye and analyzed by flow cytometry. **$p < 0.01$ compared with the control group; #$p < 0.05$, ##$p < 0.01$ compared with the gentamicin-treated group.

that incubation with gentamicin-induced apoptotic cell death. Incubation with gentamicin increased the number of Annexin V-positive cells, which was remarkably suppressed by taxifolin (Fig. 6A). Next, we performed Hoechst 33258 staining to evaluate the morphological characteristics of apoptosis, including nuclear condensation. Apoptotic cells displayed bright blue fluorescence compared to healthy cells. Compared to that in the control, the incidence of fragmented nuclei was significantly increased by treatment with gentamicin but was markedly decreased by treatment with taxifolin (Fig. 6B). Taken together, these results suggest that taxifolin significantly decreased gentamicin-induced UB/OC-2 apoptotic cell death.

4. DISCUSSION

Gentamicin is an aminoglycoside antibiotic with numerous side effects, including the possibility of irreversible ototoxicity. Taxifolin is a bioactive flavonoid that possesses therapeutic promise for the treatment of various diseases, such as cancer, liver disease, and cardiovascular diseases.[33] In this study, cochlear cells were used *in vitro* to investigate the mechanisms involved in taxifolin-induced otoprotection. We demonstrated that taxifolin could ameliorate gentamicin-induced ototoxicity. To analyze the protective effects of taxifolin against gentamicin-induced ototoxicity in UB/OC-2 cochlear cells, we investigated several possible mechanisms of action. The role of taxifolin in the mitigation of ROS generation, alleviation of mitochondrial dysfunction, and amelioration of apoptotic factors by Bcl-2, Bax, and Caspase activation was investigated.

Taxifolin is found in onions, grapes, citrus fruits, and several herbs (such as milk thistle and Douglas fir bark).[24,34] Many studies have indicated that taxifolin provides or promotes health benefits in commercial preparations such as silymarin (Legalon). Silymarin, a hepatoprotective herbal drug isolated from the seeds of milk thistle, is composed of isomeric flavolignans and a small amount of the flavonoid taxifolin.[35] Taxifolin is well known to play an antioxidant role in the effects of silymarin, which could effectively scavenge free radicals and destroy hydroxyl radicals.[36] However, taxifolin is rarely used as a single compound for disease treatment.

Flavonoids are widely present in fruits and vegetables and show strong antioxidant activity with the ability to reduce the formation of free radicals. The potent antioxidative activity of flavonoids is based on the number and position of hydroxyl groups.[37] Taxifolin is an effective antioxidant because of the presence of five phenolic hydroxyl groups in its structure.[26] Taxifolin belongs to the flavanonol subgroup of flavonoids and shows greater bioavailability than other flavonoid compounds owing to its direct absorption in the intestines in its aglycone form.[25]

P. orientalis Fructus, a traditional Chinese medicine, is rich in flavonoids, including quercetin and taxifolin, which have been shown to stimulate blood circulation and relieve pain. Quercetin and taxifolin are structurally very similar and differ only in the

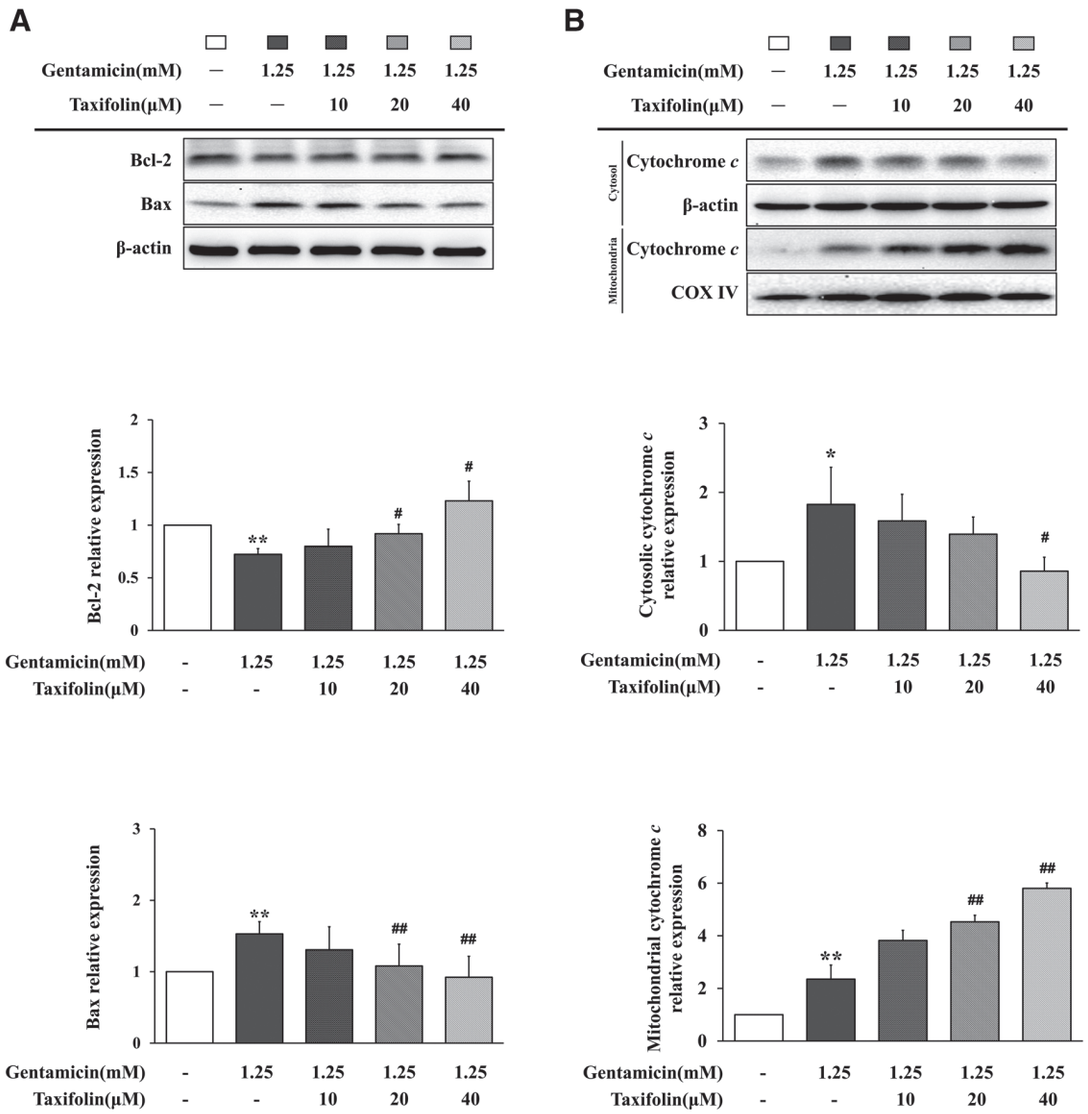

Fig. 4 Effects of taxifolin on gentamicin-induced mitochondrial apoptotic pathway. UB/OC-2 cochlear cells were pretreated with 10, 20, and 40 μM taxifolin for 2 h and co-incubated with 1.25 mM gentamicin for 48 h. A, Expression of Bcl-2 and Bax. B, Expression of cytochrome *c* in cytosolic and mitochondrial fractions. β-actin was used as the loading control for the cytosolic fraction; COX IV was used as the loading control for the mitochondrial fraction. *$p < 0.05$, **$p < 0.01$ compared with the control group; #$p < 0.05$, ##$p < 0.01$ compared with the gentamicin-treated group.

presence/absence of a C2, C3-double bond in the C-ring.[38] In taxifolin there is a single bond between C2 and C3 to form a nonplanar molecule, making it more water-soluble than quercetin at effective doses. Previous studies have demonstrated that taxifolin has neither toxic effects in toxicity studies nor any side effects *in vivo*.[39] Unlike those with quercetin, mutagenicity and genotoxicity from taxifolin have not been observed.[39,40] Thus, taxifolin might be a novel potent flavonoid with potential health benefits. In our study, taxifolin was extracted from *P. orientalis* Fructus and had little or no effect on the viability of UB/OC-2 cells (>40 μM).

Aminoglycoside antibiotics are used to treat bacterial infections because of their low price and efficacy. However, the incidence of ototoxicity and nephrotoxicity limits their use in clinical practice.[4,6,7] Damage to the vestibular and/or auditory system results in vertigo, tinnitus, and temporary or permanent hearing loss.[6] Hearing loss is irreversible because hair cells lack spontaneous regenerative capacity.[41] Recent studies using gene and stem cell therapy could possibly establish therapeutic approaches for hair cell regeneration, but overcoming challenges is necessary for functional maturation and survival time extension.[42] One possible way forward is to develop a potential drug to ameliorate aminoglycoside antibiotic-induced ototoxicity.

The mechanisms of aminoglycoside antibiotic-induced ototoxicity include numerous processes, such as the stress response, inflammation, proliferation, and cell death.[43,44] Aminoglycoside antibiotics lead to apoptotic cell death rather than necrotic cell death in animal models.[45] Cells trigger apoptotic cell death through extrinsic or intrinsic pathways. The extrinsic pathway is mediated by tumor necrosis factor superfamily receptors, which recruit Caspase-8 activation.[46] The intrinsic pathway is triggered by internal cellular stress that causes mitochondrial dysfunction, resulting in altered membrane permeability, increased cytochrome *c* release from the mitochondria to the cytosol, and activated Caspase-9.

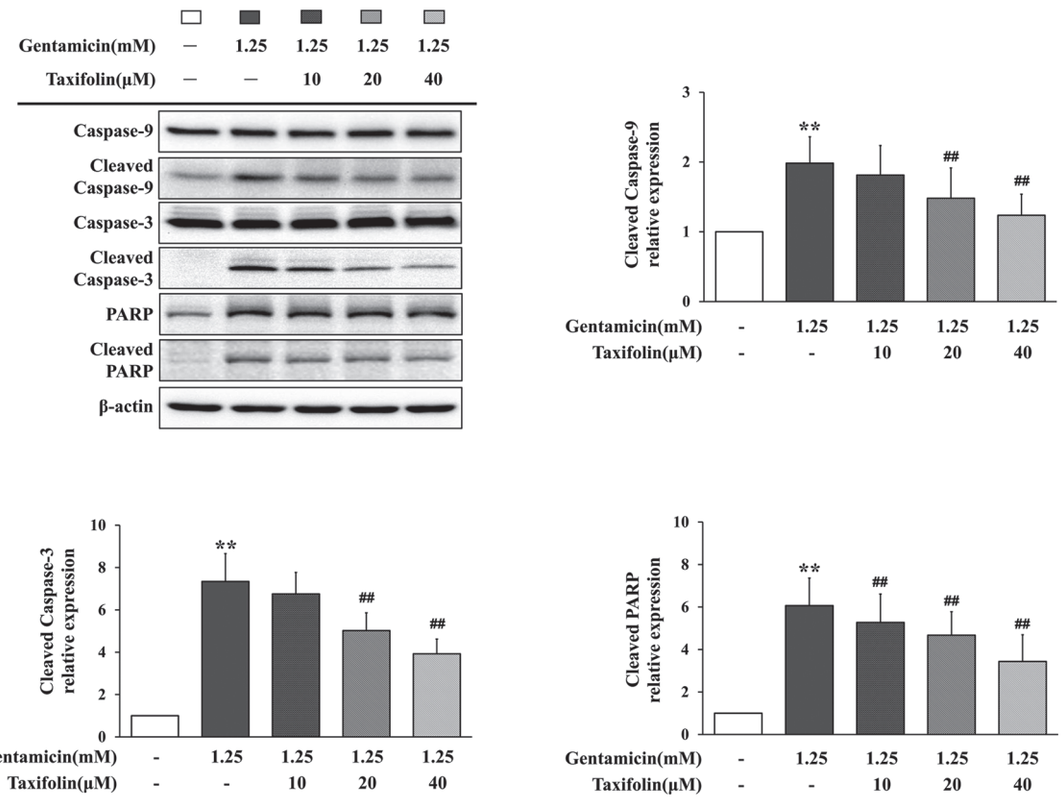

Fig. 5 Effects of taxifolin on gentamicin-induced apoptosis-related proteins. UB/OC-2 cochlear cells were pretreated with 10, 20, and 40 μM taxifolin for 2 h and co-incubated with 1.25 mM gentamicin for 48 h. The protein expression of Caspase-9, cleaved Caspase-9, Caspase-3, cleaved Caspase-3, PARP, and cleaved PARP were determined by western blot analysis. β-actin was used as the loading control. **$p < 0.01$ compared with the control group; ##$p < 0.01$ compared with the gentamicin-treated group.

The activation of Caspase-8 and Caspase-9 both promoted apoptosis by activating downstream Caspase-3. PARP, a nuclear enzyme involved in DNA repair, is inactivated by Caspase-3 cleavage and an indicator of apoptosis.[11,23,43,46] Our data showed that taxifolin pretreatment significantly mitigated cytotoxicity in UB/OC-2 cells injured by gentamicin and inhibited gentamicin-induced apoptotic cell death based on a cytotoxicity assay with LDH detection, nuclear staining with Hoechst 33258 staining, apoptotic cell staining with annexin V/PI double staining, and apoptosis-related protein expression with western blot analysis.

Several researchers have pointed out that aminoglycoside antibiotic exposure alters the redox balance, resulting in high free radical production rates.[23] Antioxidant compounds can be classified into three groups according to their mechanism as follows: (1) binding to ROS and preventing ROS-mediated protein oxidation and lipid peroxidative and DNA damage; (2) binding molecules involved in ROS formation; (3) playing a part in endogenous antioxidant production or in antioxidant recycling.[47,48] The idea of antioxidants preventing ototoxicity will lead to safety benefits and an affordable drug price. Studies have shown that N-acetylcysteine, a free radical scavenger and a precursor of glutathione, ameliorates aminoglycoside-induced ototoxicity by regulating the oxidative/antioxidant cellular balance.[47,49,50] Gentamicin-treated end-stage renal disease patients with hemodialysis who received N-acetylcysteine had a significantly decreased risk of ototoxic effects.[50] Previous study has demonstrated that N-acetylcysteine could offer hair cell protection from gentamicin-induced damage *in vitro* culture of cochlear explants by increasing intracellular glutathione level.[51] *In vivo* study showed that N-acetylcysteine had a protection effect of the cochlea against gentamicin ototoxicity via reduction of ROSs.[52] Antioxidants, such as iron chelators, calcium antagonists, and glutamate receptor antagonists, are known for their ability to participate in ROS generation.[48] D-methionine significantly reduced gentamicin-induced free radical formation *in vitro* and attenuated gentamicin-induced threshold shifts in guinea pigs *in vivo*.[14] In zebrafish, D-methionine treatment had potential protective effects against gentamicin damage on cell death signaling cascades in hair cells of the lateral line.[53] D-Methionine affected cisplatin ototoxicity by activating endogenous antioxidant signaling.[49,54] Other molecules with radical-scavenging ability might protect against ototoxicity, such as vitamin E, serotonin, plant extracts containing phenols, and flavonoids compounds.[48] Vitamin E has been shown to protect outer hair cell function from gentamicin ototoxicity by reducing gentamicin-induced free radical formation in albino guinea pigs.[55] Our data showed that taxifolin pretreatment could significantly alleviate ROS production induced by gentamicin. Future studies should investigate the antioxidant mechanisms through which taxifolin prevents ototoxicity.

Previous studies have demonstrated that complexes of aminoglycoside antibiotics interact with metal ions to generate free radicals via metal-catalyzed oxidation, which is the initiator of the apoptotic cascade.[46,56] Bcl-2 family members are regulators of cell survival and cell death in the mitochondria, and the ratio of the anti-apoptotic protein Bcl-2 to the pro-apoptotic protein Bax is decreased during apoptotic cell death.[46,57,58] Bcl-2 family proteins are involved in maintaining the integrity of the mitochondrial membrane with the intrinsic pathway and contribute to Caspase-3 activation.[46,58] Gentamicin accumulates in the

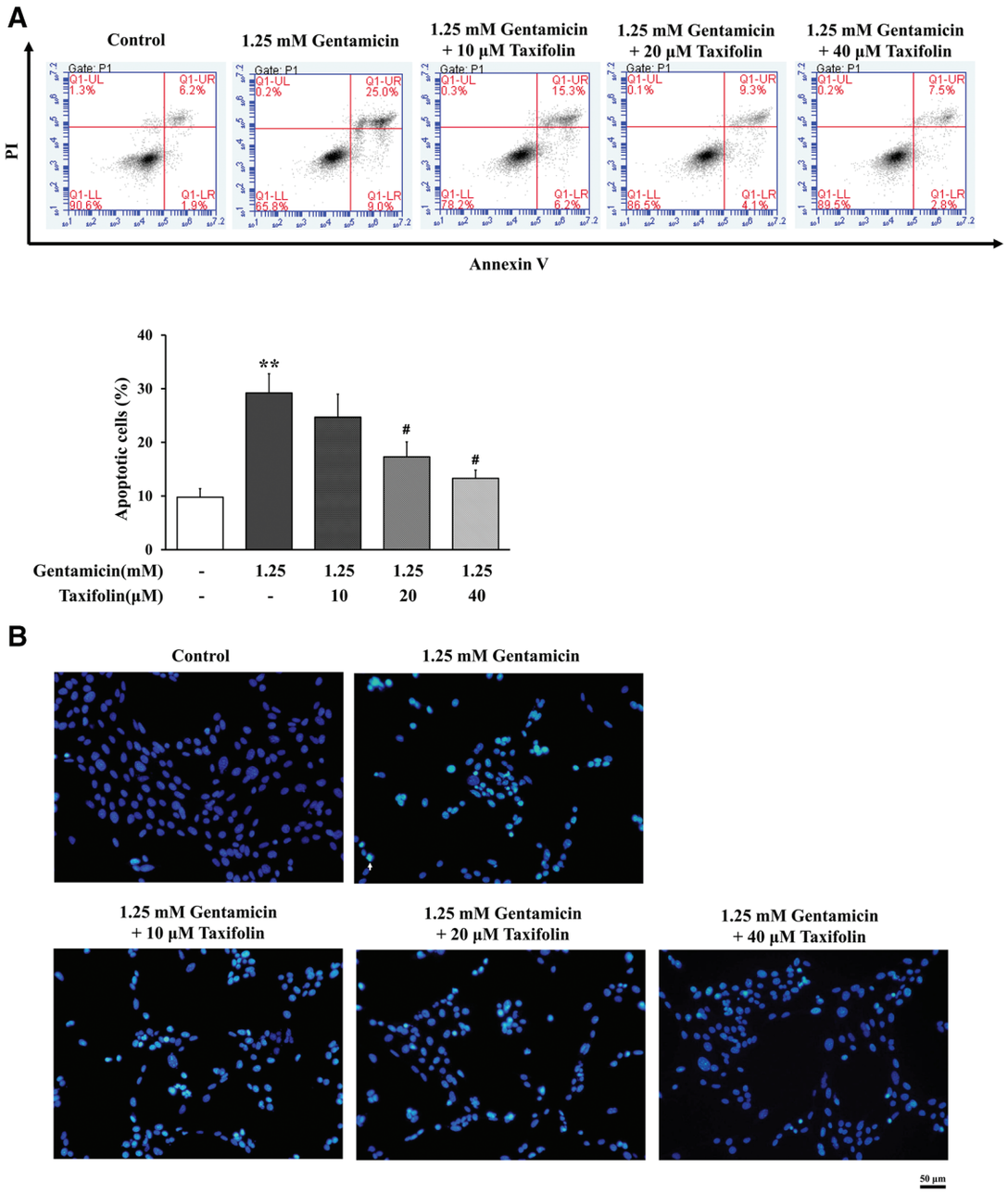

Fig. 6 Effects of taxifolin on gentamicin-induced apoptotic cell death and nuclear condensation. A, Apoptotic cell was detected using Annexin V and propidium iodide (PI) dye and analyzed by flow cytometry. $^{**}p < 0.01$ compared with the control group; $\#p < 0.05$ compared with the gentamicin-treated group. B, Nuclei were stained using Hoechst 33258 dye and visualized under a fluorescence microscope at 200× magnification. Scale bar = 50 μm.

mitochondria of hair cells and promotes ROS production, which then leads to mitochondrial permeability transition pore-opening potential.[22] To explore the underlying mechanism, we tested the hypothesis that gentamicin-induced cell death is mediated by the mitochondrial apoptotic pathway. We demonstrated that mitochondria were damaged by gentamicin exposure by detecting the loss of mitochondrial membrane potential, the release of cytochrome *c* from the mitochondria to the cytosol, the downregulation of Bcl-2, and the upregulation of Bax, whereas taxifolin was observed to reverse these effects. In summary, the mitochondrial-mediated apoptotic pathway was investigated to elucidate the possible signaling mechanisms associated with the effect of taxifolin on gentamicin-induced ototoxicity. These findings provide insights into the pharmacological effects of taxifolin, which has potential as a new candidate for clinical therapy to alleviate gentamicin-induced ototoxicity.

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