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Pinocembrin Attenuates Gentamicin-induced Nephrotoxicity in Rats

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Running title: Pinocembrin Ameliorates Gentamicin Nephrotoxicity

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ABSTRACT

Oxidative stress-mediated apoptosis of renal tubular cells is a major pathology of gentamicin-induced nephrotoxicity which is one of the prevailing causes of acute renal failure. Pinocembrin is a major flavonoid found in rhizomes of fingerroot (*Boesenbergia pandurata*). It has pharmacological and biological activities including antimicrobial, anti-inflammatory, and antioxidant effects. Preclinical studies have suggested that pinocembrin protects rat brain and heart against oxidation and apoptosis induced by ischemia-reperfusion model. The aim of the current study was to investigate the mechanisms of renoprotection elicited by pinocembrin in gentamicin-induced nephrotoxicity. Nephrotoxic rats were induced by intraperitoneal injection (i.p) of gentamicin and pinocembrin was administered via i.p. 30 min before gentamicin treatment for 10 days. Gentamicin-induced nephrotoxicity was indicated by the reduced renal function and renal Oat3 function and expression. Gentamicin treatment also stimulated Nrf2, HO-1, and NQO1 and the pro-apoptotic protein, Bax and caspase-3, concomitant with the attenuation of Bcl-XL expressions in the renal cortical tissues. Pinocembrin pretreatment improved renal function, renal Oat3 function, reduced oxidative stress and apoptotic conditions. These findings indicate that pinocembrin has a protective effect against gentamicin-induced nephrotoxicity which may be due in part to its antioxidant and anti-apoptotic effects, subsequently leading to improved renal function.

Keywords: Pinocembrin**;** Nephrotoxicity; Renal function; Organic anion transporter; Gentamicin; Oxidative stress; Apoptosis

Introduction

Gentamicin, an aminoglycoside antibiotic, has been widely used as a bactericidal agent against severe gram-negative infections (Edson and Terrell 1999; Noone et al. 1974). However, prolonged treatment of gentamicin produces serious side effects such as nephrotoxicity and ototoxicity (Lopez-Novoa et al. 2011; Rizzi and Hirose 2007). Gentamicin-induced renal toxicity is related to its preferential accumulation in the renal proximal convoluted tubules (Abdel-Raheem et al. 2009), leading to the damage of tubular epithelial cell, which further progresses to acute renal failure (Nagai and Takano 2004). Although the mechanisms of gentamicin-induced nephrotoxicity are not fully defined, the generation of reactive oxygen species (ROS), mostly in the renal cortical mitochondria (Walker and Shah 1987; Yang et al. 1995), induced vasoconstriction, mesangial cell contraction, cellular damage and necrosis via lipid peroxidation, and these changes could be prevented or ameliorated by antioxidants (Abdel-Raheem et al. 2009; Ajami et al. 2010; Mazzon et al. 2001; Nasri et al. 2013).

Nuclear factor E2-related factor-2 (Nrf2), a redox-sensitive transcription factor, is a sensor of oxidative and electrophilic stress (Xing et al. 2012). In *in vitro* and *in vivo* studies have shown that Nrf2 is essential for the antioxidant response element (ARE)-mediated induction of several genes including phase II detoxifying enzymes such as glutathione-S-transferase and quinine reductase (Kalayarasan et al. 2009). Nrf2 also activates the antioxidant enzymes and many other proteins that detoxify xenobiotics and neutralize ROS and/or reactive nitrogen species (RNS). In gentamicin-induced ototoxicity, Nrf2 protected hair cell damage by activating Nrf2-mediated antioxidant enzymes including NAD(P)H dehydrogenase quinine 1 (NQO1), GCLC (glutamate-cysteine ligase catalytic subunit), SOD, and hemeoxygenase 1 (HO1) (Hoshino et al. 2011).

Organic anion transporter (Oat) plays a major role in the elimination of organic anion substance. It is a family of solute carrier (SLC) transporter that is classified into many types such as Oat1, Oat2, Oat3, Oat4, Oat5, Oat8, Oat9 and Oat10 (Koepsell 2013; Sekine et al. 2000). Oat3 shows the highest expression at basolateral membrane of proximal tubule cell. An impaired renal excretion of various compounds along with

down-regulation of Oat3 has been reported in gentamicin-induced acute renal failure (Guo et al. 2013). However, the mechanisms of these alterations have not been clearly elucidated.

Pinocembrin (5,7-dihydroxyflavonone, $C_{15}H_{12}O_4$), a flavonoid found abundantly in honeybee propolis (Bankova et al. 1982) and the rhizomes of *Boesenbergia pandurata* (Punvittayagul et al. 2011), has several biological actions including anti-microbial (Del Rayo Camacho et al. 1991; Pepeljnjak et al. 1985), antioxidant (Santos et al. 1998), anti-inflammatory (Sala et al. 2003; Soromou et al. 2012), and vaso-relaxation (Shi et al. 2011) effects. Recently, preclinical studies have suggested pinocembrin attenuates cerebral ischemic injury in middle cerebral artery occlusion rats, (Gao et al. 2008). Pinocembrin also reduced compensatory increase in superoxide dismutase (SOD) activity, decreased in both malondialdehyde (MDA) content and myeloperoxidase (MPO) activity in global cerebral ischemic/reperfusion (I/R) rat models (Shi et al. 2011). It exhibited cardioprotective effects during I/R by its antioxidant and anti-apoptotic effects (Lungkaphin et al. 2015). These data led to investigate the renoprotective effect of pinocembrin against gentamicin-induced nephrotoxicity. We tested the hypothesis that the renoprotective mechanisms of pinocembrin against gentamicin-induced nephrotoxicity due to its antioxidant and anti-apoptotic effects.

Materials and methods

Chemicals and reagents

Gentamicin was acquired from The Govt. Pharm.Org (Bangkok, Thailand).The pinocembrin compound (95% purity) was isolated from the rhizomes of *Boesenbergia pandurata* which was carried out at the Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Lampang Rajabhat University (Lampang, Thailand). Tween 80 was supplied by Calbiochem, Merck Millipore (Billerica, MA, USA)**.** Mammalian tissue lysis/extraction reagent was provided by Sigma Chemical Co (MO, USA). Complete protease inhibitor cocktail was acquired from Roche Applied Science (IN, USA)**.** TBARS assay kit was purchased

from Cayman Chemical (Ann Arbor, MI, USA). SOD activity assay kit was provided by BioAssay Systems (CA, USA). BUN and creatinine assay kits were purchased from DiaSys Diagnostic Systems GmbH (Holzheim, Germany). The radiolabeled estrone sulfate ([^{3}H]ES) was purchased from PerkinElmer (TX, USA). Primary Oat3 antibody was from Cosmo Bio Co. Ltd. (Tokyo, Japan). The Na^+-K^+-ATPase, caspase-3 and Bcl-XL antibodies were acquired from Millipore (MA, USA). The primary anti-Heme Oxygenase 1 (HO-1) and primary anti-NQO1 antibodies were from Abcam (MA, USA). The primary PKCα, NADPH oxidase (NOX4) and primary anti-Nrf2 antibodies were from Santa Cruz Biotechnology (TX, USA). The primary anti-Bax, and β-actin antibodies were from Cell Signaling Technology (MA, USA). The horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody was purchased from Amersham (IL, USA). A poly-vinylidene fluoride (PVDF) membrane was provided by Millipore (MA, USA). The ECL enhanced chemiluminescence agent and Hyperfilm were acquired from GE Healthcare (Buckinghamshire, UK).

The method of pinocembrin preparation

The air-dried powder (1 kg) of *Boesenbergia pandurata* rhizomes was percolated with *n*-hexane at room temperature for 9 days. Subsequently, the residue was percolated with ethyl acetate for 15 days. Then, the filtrate was evaporated to dryness under low pressure to obtain anethyl acetate crude extract (79.99 g). To obtain pinocembrin, the ethyl acetate extract was firstly subjected to coarse separation on a silica column chromatography. Gradient elution was conducted initially with *n*-hexane, gradually enriched with ethyl acetate, followed by increasing amounts of methanol in ethyl acetate and finally with methanol. The obtained solid was recrystalized from ethanol to obtain pinocembrin (69.32 mg). Finally, structural confirmation was performed using UV, FTIR, $_1$H NMR, $_{13}$C NMR, MS. The purity of pinocembrin was more than 95% (Charoensin et al. 2010).

Previous study investigated the toxicity dose of pinocembrin in a rat model (Charoensin et al. 2010). Pinocembrin at the doses of 1-100 mg/kg and 500 mg/kg were administrated by gavage feeding. There were neither toxic nor death in rats studied in

that model. Recently, there was a report in a double-blind, placebo-controlled, randomized study carried out in 58 healthy subjects (Cao et al. 2015). Single ascending doses of pinocembrin (20–150 mg) as well as multidose study at 60-mg pinocembrin were investigated. The results showed that pinocembrin was well tolerated and no serious adverse events occurred. No subjects were discontinued because of a treatment emergent AE. These findings indicated that there was no lethal or toxic dose of pinocembrin.

Animals

Male Sprague-Dawley rats (240-250 g) from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom were housed in the animal room at controlled temperatures in a 12:12 h light/dark cycle and fed with a normal pellet diet and water ad libitum. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine, Chiang Mai University (Permit Number: 13/2557). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Experimental design

The rats were randomly divided into 5 groups (6 rats per group) and treated for 10 days, as follows:- (1) Control group: the rats were injected intraperitoneally (i.p.) with Tween 80, (2) Gentamicin group: the rats were treated (i.p.) with gentamicin at a dose of 100 mg/kg/day, (3) Pinocembrin plus Gentamicin group: the rats were injected (i.p.) with pinocembrin (dissolved in Tween 80 at a dose of 50 mg/·kg/day, obtained from air-dried finger-root weighing 140 g) 30 min prior to the injection of gentamicin, (4) Pinocembrin-50 group: the rats were injected i.p. with pinocembrin (50 mg/kg/day) for 10 days and (5) Pinocembrin-75 group: the rats were injected i.p. with pinocembrin (75 mg/kg/day) for 10 days. The dose of pinocembrin used in this study was chosen from our preliminary experiment and from a previous study (Soromou et al. 2012).

After the treatment on 10th day, the animals were placed into individual metabolic cage for 24 h urine collection and then sacrificed under anesthesia for blood collection from the right atrium. The kidneys were immediately removed, decapsulated, and weighed. One of the kidneys was divided into two longitudinal sections. Renal cortical tissues were isolated and kept for western blot analysis and evaluation of MDA. The other kidney was perfused with cold PBS, and then cut into two longitudinal sections; one half was fixed in 10% neutralized formalin for further morphological analysis and the other half, the renal cortical tissues were isolated and kept for SOD determination. Then, the tissue samples were placed in liquid nitrogen and stored at -80 ºC until use.

Determination of renal function

The serum and urine creatinine and serum BUN levels were measured by following enzymatic colorimetric methods using commercial kits. The data were expressed as mg/dL.The estimation of glomerular filtration rate (GFR) or creatinine clearance (C_{cr}) was carried out using the following equation:

$$C_{cr}\ (mL/min) = \frac{\text{urine creatinine X urine flow rate}}{\text{Serum creatinine}}$$

Determination of renal Oat3 function

The uptake of radiolabeled estrone sulfate ([^{3}H]ES), a specific Oat3 substrate, into the renal cortical slice, which reflects the renal Oat3 function, was examined. After the animals were sacrificed, the kidneys were removed, decapsulated and placed in freshly oxygenated ice-cold modified Cross and Taggart saline buffer (containing the following: 95 mM NaCl, 80 mM mannitol, 5 mM KCl,0.74 mM $CaCl_2$, and 9.5 mM Na_2HPO_4, pH 7.4). Thin renal cortical slices (≤ 0.5 mm; 5-15 mg/slice, wet weight) were cut with a Stadie-Riggs microtome and were pre-incubated in modified Cross and Taggart buffer for 10 min then incubated in 1 mL of buffer containing 50 nM [^{3}H]ES for 30 min at room temperature. At the end of the uptake period, the slices were washed in 0.1 M $MgCl_2$, blotted on filter paper, weighed, and dissolved in 0.5 mL of 1 M NaOH

and then the preparation was neutralized with 0.5 mL of 1N HCl. The radioactivity was measured using a liquid scintillation analyzer (PerkinElmer, MA, USA). The [^{3}H]ES uptake was calculated as tissue to medium (T/M) ratio (dpm/g tissue÷dpm/mL medium).

Tissue preparation for western blot analysis

Renal cortical tissue, 0.1 g, was chopped and homogenized on ice in Mammalian cell Lytic buffer with a protease inhibitor cocktail. Each cellular component, whole cell lysate, membrane, and cytosolic fraction, were prepared from renal cortical slices using differential centrifugation as previously described (Lungkaphin et al. 2014). Briefly, the homogenate was centrifuged at 5,000x g for 10 min at 4 ºC, the supernatant was designated as whole cell lysate, and then the supernatant was further centrifuged at 100,000x g for 2 h at 4 ºC to obtain a membrane (pellet) and cytosolic (supernatant) fractions. The 5,000x g pellet was re-suspended and centrifuged at 10,000x g 4 ºC for 10 min. The supernatant fraction from the spin was designated as the nuclear fraction. All the fractions collected were stored at -80 ºC until use.

Determination of renal Oat3 expression

The total cell lysates and the membrane fractions from the renal cortex were subjected to SDS-PAGE, and subsequently transferred to a PVDF membrane, as described above. Primary Oat3 antibody at concentration of 1:500 was added. To confirm the enrichment of the membrane fraction, the Na^+-K^+-ATPase expression was determined as a membrane fraction marker. The density of the protein signal on Hyperfilm was analyzed using the histogram function of Adobe Photoshop CS5 (Adobe Corp., CA, USA) scanning. The protein level was normalized by β-actin as a loading control.

Determination of renal lipid peroxidation

In order to determine conditions of renal oxidative stress, the measurement of malondialdehyde (MDA) level, a marker of lipid peroxidation, in the renal cortical

tissues was carried out. Briefly, the renal cortical tissues were cut and suspended in CelLyticMT mammalian tissue lysis/extraction reagent containing a 1% complete protease inhibitor cocktail, made according to the manufacturer's protocol. The tissues were then homogenized and centrifuged at 1,600 g for 10 min at 4 °C. The supernatants were collected for the determination of MDA concentration using a commercial TBARS assay kit, as previously described (Ohkawa et al. 1979). Each sample was expressed as total MDA level to total protein concentration (nmol/mg protein).

Determination of renal cortical superoxide dismutase (SOD) activity

The renal cortex tissue was homogenized at 5 mL/g in cold lysis buffer (50 mM potassium phosphate, 0.1 mM EDTA, 0.5% Triton X-100) and centrifuged at 12,000 g for 5 min at 4°C. The supernatants were used for total SOD activity determination, according to the manufacturer's protocol.

Determination of renal oxidative stress and apoptosis

The renal oxidative stress pathway and apoptosis protein markers were determined by western blot analysis. The renal cortical fraction was used to determine the protein expression of PKCα, Nrf2, heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase quinine 1 (NQO1), NADPH oxidase (NOX4), Bax, Bcl-XL and caspase-3. The whole cell fraction was used to determine the PKCα, HO-1, NQO1, NOX4, caspase-3 and the Bcl-2 protein family (pro-apoptotic; Bax and anti-apoptotic; Bcl-XL proteins) expressions by western blot analysis as described above. Briefly, total cell lysates, cytosolic, membrane and nuclear fractions from the renal cortex were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to a poly-vinylidene fluoride (PVDF) membrane. The primary PKCα antibody at concentration of 1:2,000, primary anti-Heme Oxygenase 1 (HO-1) antibody at concentration of 1:2000, primary anti-NQO1 antibody at concentration of 1:500, primary anti-NOX4 antibody at concentration 1:500, primary anti-Bax at concentration of 1:3000, Bcl-XL at concentration of 1:500 and caspase-3 at concentration 1:250 were used to probe overnight at 4°C. For determination of the Nrf2

expression was performed using nucleus and cytosol fractions. Primary anti-Nrf2 antibody at concentration of 1:250 was added. The membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody at room temperature for 1 h and developed with an ECL enhanced chemiluminescence agent. Each membrane was stripped and re-probed with mouse anti-β-actin antibody that served as a loading control or other antibody for further detection of the interest protein expression. The densities of the protein signals on the Hyperfilm were analyzed using the histogram function of Adobe Photoshop CS5 (Adobe Corp., CA) scanning.

Histopathological study

The paraffin-embedded specimen was cut into 2 μm-thick sections, mounted on microscope slides, and stained with hematoxylin and eosin (H&E) for histological assessment. The samples were examined under a light microscope for evaluation of tubular and glomerular changes. Histopathological alteration or tubular damage was assessed by the degree of tubular dilatation, necrosis, apoptosis and cast formation in the renal tubular cells.

Statistical analysis

The data are expressed as mean ± standard error of mean (S.E.M) and analyzed using the SPSS version 17 statistical program (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA), followed by the Newman-Keuls test, was performed. A P value < 0.05 was considered statistically significant.

Results

The effect of pinocembrin pretreatment on physiological and renal function parameters in the gentamicin-induced nephrotoxicity

There was no difference in the mean initial body weight between the experimental groups. After 10 day of treatment, the gentamicin-treated rats had significantly lower body weights, and markedly higher kidney weight as well as higher

kidney weight per body weight ratio than those of the control rats ($P< 0.05$) (Table 1). Compared with the control group, the serum BUN and creatinine levels were significantly higher ($P< 0.05$) while the C_{cr} was markedly lower in the gentamicin group ($P< 0.05$) indicating impaired renal function. Pinocembrin pretreatment (50 or 75 mg/kg) apparently increased the body weight and decreased the kidney weight and kidney weight per body weight ratio ($P< 0.05$). The serum BUN and creatinine levels were also significantly decreased ($P< 0.05$) although the C_{cr} had a tendency to increase, in gentamicin plus pinocembrin (50 or 75 mg/kg) group compared with the gentamicin group with pinocembrin. Since the pinocembrin at the doses of 50 and 75 mg/kg showed similar results, we selected to use pinocembrin at the low dose (50 mg/kg) for the subsequent experiments.

The effect of pinocembrin pretreatment on renal Oat3 function and expression in the gentamicin-induced nephrotoxicity

A significant decrease in the [^{3}H]ES uptake into the renal cortical slides was observed in the gentamicin-treated rats compared to that of the control rats ($P< 0.05$) (Fig. 1). Interestingly, pinocembrin pretreatment led to significantly improved the renal Oat3 function as shown by an increase in the [^{3}H]ES uptake compared with the gentamicin group ($P<0.05$). To determine whether the decreased function of renal Oat3 in the gentamicin-treated rats was partly due to the down-regulated expression of Oat3 at the basolateral membrane, the Oat3 expression in the membrane and whole cell lysate fractions of the renal cortex were determined by the western blot analysis. The expression level of renal Oat3 from the whole cell lysate fraction was unchanged in all the experimental groups (Fig. 2B). However, the membrane expression of Oat3 in the gentamicin-treated rats was significantly decreased when compared with the control group ($P< 0.05$) (Fig. 2A). Pinocembrin pretreatment significantly attenuated a decreased membrane expression of Oat3 when compared to the gentamicin-treated rats ($P< 0.05$). These results suggest that the decreased renal Oat3 function in the gentamicin-treated rats may result from the down-regulation of Oat3 at the membrane of renal tubular cells. The reduced renal Oat3 function and expression can be improved by pinocembrin pretreatment in this study.

The effect of pinocembrin pretreatment on oxidative stress conditions in the gentamicin-induced nephrotoxicity

The renal cortical MDA was increased in the gentamicin-treated rats in relation to that of the control rats ($P< 0.05$) (Fig. 3A). An apparent decrease of MDA to normal level in the pinocembrin plus gentamicin group ($P< 0.05$) indicated that a marked generation of oxidative stress by gentamicin is significantly prevented by pinocembrin pretreatment. The result of SOD activity was consistent with the previous studies, demonstrating that gentamicin-treated rats had significant decrease in the SOD activity as compared with the control rats ($P< 0.05$) (Fig. 3B). Surprisingly, pinocembrin pretreatment could not improve the activity of the SOD enzyme when compared with the gentamicin-treated group.

The effect of pinocembrin pretreatment on the oxidative stress pathways in the gentamicin-induced nephrotoxicity

Based on previous findings that PKCα was activated by the overproduction of ROS, we determined whether an increased oxidative stress in the renal cortical tissue by gentamicin could activate PKCα. As shown in Fig. 4A, the gentamicin-treated rats significantly enhanced PKCα expression when compared with the control rats ($P< 0.05$), and this increased PKCα expression was significantly reduced by pinocembrin pretreatment ($P< 0.05$). These findings indicate that the overproduction of ROS in gentamicin-treated rats activates PKCα signaling pathways, and pinocembrin can attenuate the production of ROS and consequently inactivate PKCα. We found that NOX4 expression was stimulated in gentamicin-treated rats ($P< 0.05$). The treatment with pinocembrin could inhibit NOX4 expression as compared to the gentamicin-treated rats ($P< 0.05$) (Fig. 4B).

Nrf2, the transcription factor that promotes the antioxidant defense system or protects against oxidative stress, has been shown to protect against gentamicin-induced hair cell damage. Thus, we postulated that an increased oxidative stress in gentamicin-treated rats may activate the Nrf2 and Nrf2-mediated antioxidant enzymes. As shown in Fig. 5A and B, the Nrf2 expression in the nuclear fraction of the renal cortical tissue

was significantly increased in the gentamicin-treated rats when compared with the control rats ($P< 0.05$). However, there was no change in the Nrf2 expression in the cytosol fraction between the experimental groups. These results suggest that the activation of Nrf2 leads to an increased translocation of Nrf2 from the cytoplasm to the nucleus in gentamicin-treated rats. Interestingly, the nuclear expression of Nrf2 was reduced ($P< 0.05$) in the pinocembrin plus gentamicin-treated rats compared to the gentamicin-treated rats. Additionally, the expressions of the antioxidant enzyme and the detoxification gene, HO-1 and NQO1, respectively, were apparently increased ($P< 0.05$) in the gentamicin-treated rats as compared with the control rats (Fig. 5C and D). Importantly, the increased HO-1 and NQO1 expressions were significantly reduced by pinocembrin pretreatment ($P< 0.05$). These results suggest that pinocembrin pretreatment can lessen the oxidative stress conditions induced by gentamicin through the modulation of the antioxidant defense parameters.

The effect of pinocembrin on renal apoptosis in the gentamicin-induced nephrotoxicity

The gentamicin-treated rats demonstrated an increase in the expression of the pro-apoptotic protein, Bax, along with a decreased expression of the anti-apoptotic protein, Bcl-XL when compared with the control rats ($P< 0.05$) (Fig. 6A, B and C). Pinocembrin pretreatment significantly reversed an altered expression of the apoptosis-related protein in the gentamicin-treated rats ($P< 0.05$). We found that caspase-3 expression was increased in the gentamicin-treated rats ($P< 0.05$). Pinocembrin treatment could reverse this effect by reducing the level of caspase-3 as compared to the gentamicin-treated rats ($P< 0.05$) (Fig. 6D).

The effect of pinocembrin on renal morphology in the gentamicin-induced nephrotoxicity

The histological changes and the pathological manifestations of the kidney are presented in Fig. 7. Normal kidney morphology was observed in the control (A) and the pinocembrin (D) groups. Nephrotoxicity in the gentamicin-treated rats was evidenced by tubular dilatation, tubular epithelial damage, intracellular cast formation, nuclear

irregularity, karyorrhexis and inflammation (B); however, these defects were ameliorated by pinocembrin pretreatment (C).

Discussion

The present study demonstrated that gentamicin treatment caused nephrotoxicity which was manifested by marked increases in serum BUN and creatinine with a decrease in C_{cr}. These findings were correlated with the histopathological damages of the kidney. The impaired renal function was accompanied with the reduced renal Oat3 function, an indicator of proximal tubular transport function. The down-regulation of renal Oat3 function and expression in gentamicin-treated rats was associated with the increases in oxidative stress and apoptosis. Pinocembrin pretreatment showed the marked decreases in ROS production and apoptosis leading to an improvement of renal function.

In this study, an elevation of renal cortical MDA level along with the decrease activity of SOD indicated the increased oxidative stress condition induced by gentamicin in rat kidneys. Several investigators have reported the relationship between free radical formation and gentamicin-induced acute renal injury (Karahan et al. 2005; Shin et al. 2014; Walker et al. 1999). The subsequent generation of reactive oxygen metabolites damages the protein molecules and degrades the membrane bound phospholipids through the process of lipid peroxidation (Sahu et al. 2013), which were correlated with the inactivation of antioxidant enzymes such as GSH-Px, CAT and SOD (Kang et al. 2013; Karahan et al. 2005). The decreased renal cortical SOD activity in gentamicin-treated rats implied the depletion of antioxidant enzymes during the combating process to oxidative stress (Kang et al. 2013). We postulated that gentamicin-induced renal injury was caused by free radical generation with an attenuation of the antioxidant enzymes. Importantly, a marked reduction of renal cortical MDA level accompanied with an improved renal function in pinocembrin plus gentamicin-treated rats might indicate that the ROS was scavenged, and lipid peroxidation was reduced by pinocembrin. However, pinocembrin pretreatment could not restore the decreased activity of SOD enzyme in gentamicin-treated rats. We

hypothesized that pinocembrin might induce other mechanisms to overdrive ROS overproduction.

The disruption of Nrf2/Keap1 complex (Keap1 is the regulatory protein of Nrf2) has been activated by oxidative stress and electrophiles (Xing et al. 2012). PKCα was activated by the overproduction of ROS (Arjinajarn et al. 2014; Lee et al. 2003). In this study, Nrf2 was activated in the gentamicin-induced nephrotocixity by the increase of oxidative stress condition indicated by the increased renal PKCα and NOX4 expressions. The activation of Nrf2 acts as a cellular adaptive response to stimulate the expression of antioxidant enzymes at specific anti-oxidant response elements (ARE) within the regulatory regions of responsive genes (Itoh et al. 1997; Kobayashi and Yamamoto 2005; Li and Kong 2009) against gentamicin-induced oxidative stress. In the present study, an increased translocation of Nrf2 into the nucleus in the renal cortical tissue of the gentamicin-treated rats could lead to the activation of target genes expression including NQO1 and HO-1. Study in rat kidney cells revealed a protective role of Nrf2 overexpression against triptolide-induced cytotoxicity in a normal rat kidney cells (NRK-52E) through counteracting oxidative stress (Li et al. 2012). Nrf2 also protected age-related hearing injuries and gentamicin-induced ototoxicity by up-regulating antioxidant enzymes including NQO1, HO-1, SOD, and GCL and detoxifying proteins (Hoshino et al. 2011). It is noteworthy that pinocembrin pretreatment resulted in a decreased ROS production leading to the inactivation of Nrf2 as indicated by a significant reduction in the nuclear translocation of Nrf2. The attenuation of oxidative stress by pinocembrin pretreatment occurred via a decreased Nrf2-mediated transcriptional regulation as well as the NQO1 and HO-1 expressions. The cytoprotective properties of pinocembrin have been shown in chronic cerebral hypoperfusion (Guang and Du 2006) and transient global brain ischemia/reperfusion (Shi et al. 2011) in rats which are associated with reduced oxidative stress. Pinocembrin might act as both direct and indirect antioxidants, via the induction of many cytoprotective proteins, including antioxidant enzymes, and through the inactivation of Nrf2 by superimposing the overproduction of ROS, thus causing the reversal of oxidative stress conditions. There are several studies investigated the protective effects of natural compounds on gentamicin-induced nephrotoxicity. Rosmarinic acid showed

to alleviate gentamicin-induced nephrotoxicity via antioxidant activity, increases of renal GSH content and renal antioxidant enzyme activity (Tavafi and Ahmadvand 2011). Recently, curcumin also found to attenuate renal injuries in gentamicin-induced toxicity in rats (He et al. 2015; Manikandan et al. 2011; Negrette-Guzman et al. 2015).

Gentamicin-induced apoptosis as shown by the elevated cellular pro-apoptotic (Bax and caspase-3) and reduced anti-apoptotic (Bcl-XL) protein expression was consistent with the histopathological changes in gentamicin-treated rat kidneys in this study. Previously, the increased expressions of apoptotic protein Bax, cytochrome c, cleaved caspase-9 and cleaved caspase-3 with a decrease in the expression of anti-apoptotic protein Bcl-2 were observed in renal tubular cells of gentamicin-induced acute kidney injury in rats (Shin et al. 2014). Excessive ROS generated in gentamicin-induced nephrotoxicity is known to cause mitochondrial dysfunction which is an early event in the intrinsic pathway of apoptosis, resulting in morphological and functional changes (Jia et al. 2013; Morales et al. 2010). Pinocembrin provided a renoprotection by inhibiting Bax and caspase-3 overexpressions induced by gentamicin with an enhancing Bcl-XL expression, leading to the alleviation of renal tubular necrosis/damage. It was reported that the anti-apoptotic Bcl-2 family protein could protect the integrity of mitochondrial membrane by binding to the outer membrane of the mitochondria and blocking the efflux of cytochrome c (Kalkan et al. 2012; Kuwana and Newmeyer 2003). The effect of antioxidant treatment on gentamicin-induced apoptosis was reported in both in vivo and in vitro studies (Kang et al. 2013; Ojano-Dirain and Antonelli 2012). Therefore, the beneficial effect of pinocembrin on gentamicin-induced apoptosis in this study could be mediated by the antioxidant effect as the altered expressions of apoptosis-related proteins were preceded by ROS production.

In the present study, gentamicin-treated rats showed the decreased renal Oat3 function and membrane expression along with an increased PKCα protein expression. The down-regulation of membrane expression of Oat3 was related to the PKCα activation by an increase in ROS production (Arjinajarn et al. 2014). Gentamicin treatment might induce the trafficking of Oat3 from the basolateral membrane into the cytoplasm of the proximal tubular cells, resulting in a decreased membrane expression

of Oat3 and subsequently a decreased renal Oat3 function. These actions might be associated with the activation of PKCα protein expression through an increased ROS generation by gentamicin treatment. These were supported by the correlation between the decreased membrane expressions of renal Oat1 and Oat3 and the increasing level of lipid peroxidation in nephrotoxicity rats (Ulu et al. 2012). The restored function and membrane expression of renal Oat3 in gentamicin-treated rats after pinocembrin pretreatment was consistent with the previous study demonstrating that decreased renal Oat1 and Oat3 expressions and functions could be reversed after pretreatment with the antioxidant substance, JBP485 (Cyclo-trans-4-L-hydroxyprolyl-L-serine) (Guo et al. 2013). Moreover, treatment with a potent scavenger of free radicals has been reported to prevent the renal toxic effects of gentamicin via the inhibition of a PKC pathway (Parlakpinar et al. 2006). Therefore, the reduction of ROS generation in gentamicin-treated rats by pinocembrin pretreatment might inactivate PKCα which in turn up-regulated the membrane expression of Oat3, leading to improved Oat3 function and finally reversal of renal dysfunction.

Conclusion

The present results clearly show that pinocembrin can protect gentamicin-induced kidney injury via an amelioration of oxidative stress and apoptosis of renal tissues. It attenuates the increase in oxidative stress and modulates the antioxidant enzymes via the Nrf2/HO-1, NQO1 pathways, thereby leading to reduce protein-related apoptosis results in improved renal Oat3 and kidney functions. Therefore, pinocembrin could be inferred as an alternative therapeutic option to prevent gentamicin-induced nephrotoxicity.

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Conflict of Interest

The authors have no conflict of interest to disclose.

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Table 1 Effects of pinocembrin pre-treatment on physiological and renal function parameters in gentamicin-treated rats

Parameter	Control	Gentamicin 100 mg/kg	Gentamicin 100 mg/kg +pinocembrin 50 mg/kg	Gentamicin 100 mg/kg +pinocembrin 75 mg/kg	Pinocembrin 50 mg/kg
Body weight (g)	337±7.95	294±8.13 *	308±2.50 #	323±3.30 #	324±7.50
Kidney weight (g)	1.33±0.02	2.00±0.16 *	1.50±0.03 #	1.47±0.06 #	1.32±0.02
KW/BW ratio	0.004±0.0001	0.006±0.0003 *	0.005±0.0001 #	0.004±0.0002 #	0.004±0.0001
Renal function					
Serum BUN (mg%)	21.50±0.76	31.16±1.83 *	26.33±1.80 #	23.33±0.88 #	21.40±0.68
Serum Cr (mg%)	0.47±0.02	0.73±0.04 *	0.58±0.03 #	0.55±0.07 #	0.37±0.04
C_{cr} (ml/min)	2.07±0.11	1.17±0.07 *	1.35±0.08 #	1.51±0.36 #	2.40±0.16

Values are mean ± SE; (n=6 rats in each group). GM, gentamicin; KW/BW ratio, kidney weight/bodyweight ratio; BUN, blood urea nitrogen; creatinine, Cr; creatinine clearance, C_{cr}. *$P < 0.05$ compared with control; #$P < 0.05$ compared with gentamicin-treated rats.

Figure legends

Figure 1 Effects of pinocembrin pretreatment on [^{3}H]ES uptake in renal cortical slices. Renal cortical slices were incubated in buffer containing 50 nM [^{3}H]ES for 30 minutes at room temperature. The uptake was calculated as tissue/medium ratio and then converted to a mean percentage of the control. Values are expressed as the mean ± SEM from six rats (5 slices/group/animal). *$P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the gentamicin-treated group.

Figure 2 Effects of pinocembrin pretreatment on Oat3 expression in the renal cortical tissue. A: Western blot analysis of Oat3 in the membrane and B: in the whole cell lysate fractions of renal cortical tissues. The signal intensity of Oat3 in membrane and whole cell lysate fractions normalized to β-actin. Bar graphs indicate mean ± SEM (from 6 rats in each group). *$P < 0.05$ compared to the control group, $^{\#}P < 0.05$ compared to the gentamicin-treated group.

Figure 3 A. Effects of pinocembrin pretreatment on the renal cortical MDA concentration. Thiobarbituric acid reactive substances (TBARS) were measured in renal cortical tissues. B. Effects of pinocembrin pretreatment on SOD enzyme activity in renal cortical tissue. Values are the mean ± SEM. (n=6 rats in each group). *$P < 0.05$ compared to the control, $^{\#}P < 0.05$ compared to the gentamicin-treated group.

Figure 4 Effects of pinocembrin pretreatment on the expressions of PKCα (A) and NOX4 (B) in renal cortical tissues. Immunoblot analysis for PKCα and NOX4 expressions in whole cell lysate fraction of renal cortical tissue and immunostaining signal intensity of PKCα and NOX4 expressions normalized to β-actin. Bar graphs indicate mean ± SEM (n=6 rats in each group). *$P< 0.05$ compared to the control, $^{\#}P< 0.05$ compared to the gentamicin-treated group.

Figure 5 Effects of pinocembrin pretreatment on the expression of Nrf2 in the renal cortical tissue. Immunoblot analysis for Nrf2 in nuclear (A) Nrf2 in cytosolic fractions (B) HO-1 (C) and NQO1 (D) expressions of renal cortical tissues. Immunostaining signal intensity protein expressions normalized to β-actin. Bar graphs indicate mean ±

SEM (n=6 rats in each group). *$P < 0.05$ compared to the control, $^{\#}P < 0.05$ compared to the gentamicin-treated group.

Figure 6 Effects of pinocembrin pretreatment on the expression of apoptotic proteins in renal cortical tissue. (A), (B) and (D): Representative immunoblot analysis for Bax, Bcl-XL and caspase-3 expressions in renal cortical tissues, respectively. (C): Immunostaining signal intensities of Bax/Bcl-XL ratio. Immunostaining signal intensities of Bax, Bcl-XL and caspase-3 expressions normalized to β-actin. Bar graphs indicate mean ± SEM (n=6 rats in each group). *$P< 0.05$ compared to the control, $^{\#}P< 0.05$ compared to the gentamicin-treated group.

Figure 7 Hematoxylin and eosin (H&E) stain of the kidneys (magnification, x40). Panels A, B, C and D are images of glomeruli and renal tubules from control (A), gentamicin (B), gentamicin + pinocembrin (C) and pinocembrin rats (D), respectively. The mitosis and tubular detachment (B) in gentamicin kidney are shown with arrowheads and black arrows, respectively.

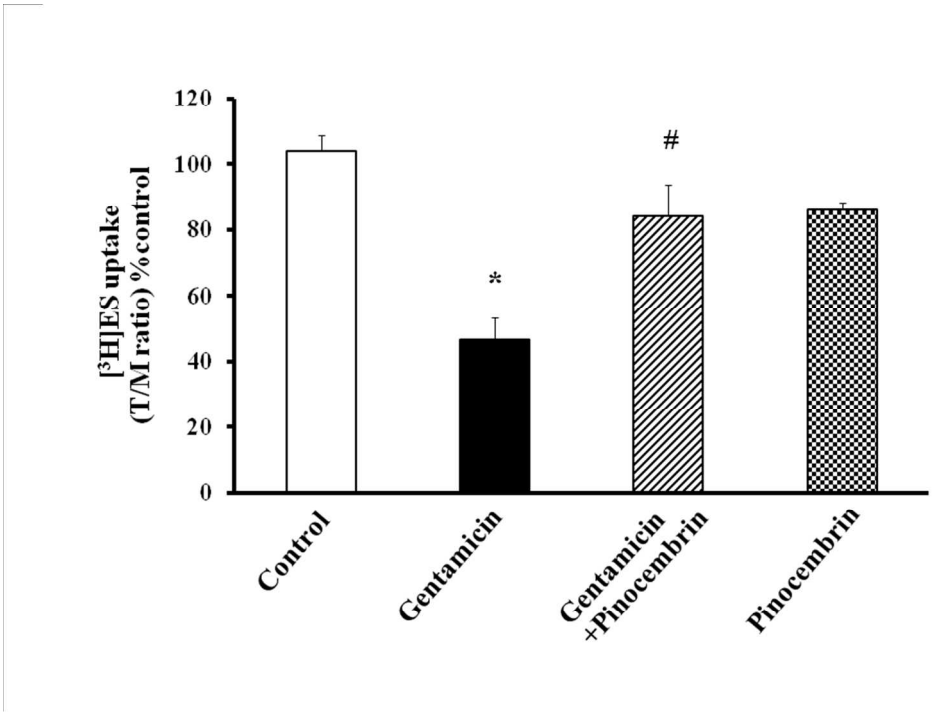

Figure 1 Effects of pinocembrin pretreatment on [3H]ES uptake in renal cortical slices. Renal cortical slices were incubated in buffer containing 50 nM [3H]ES for 30 minutes at room temperature. The uptake was calculated as tissue/medium ratio and then converted to a mean percentage of the control. Values are expressed as the mean ± SEM from six rats (5 slices/group/animal). *P < 0.05 compared to the control group, #P < 0.05 compared to the gentamicin-treated group.
420x297mm (300 x 300 DPI)

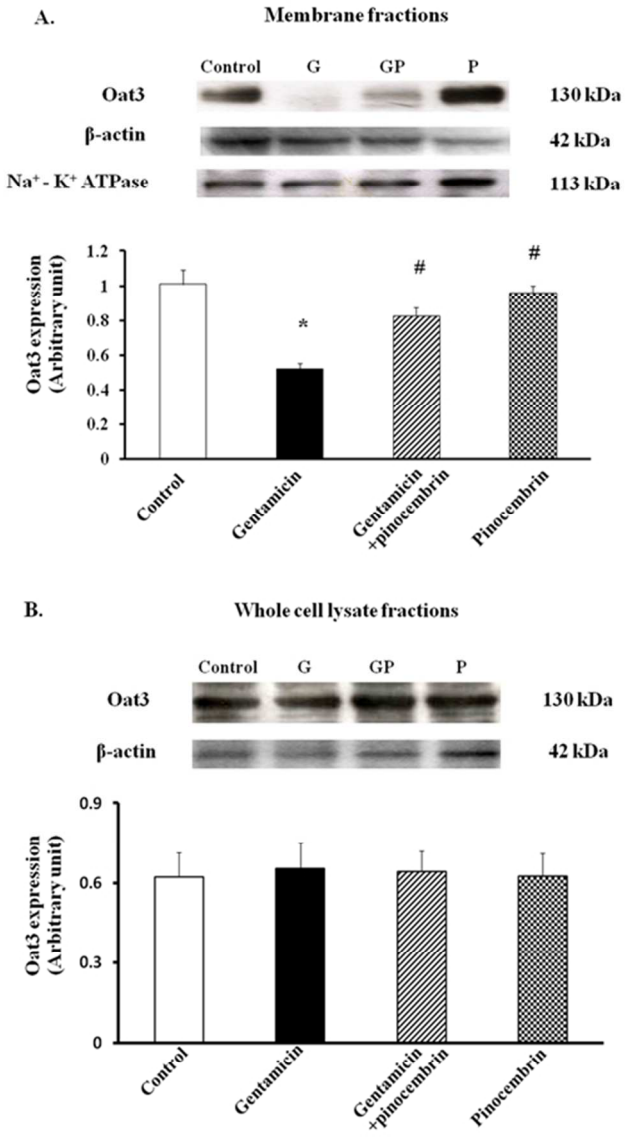

Figure 2 Effects of pinocembrin pretreatment on Oat3 expression in the renal cortical tissue. A: Western blot analysis of Oat3 in the membrane and B: in the whole cell lysate fractions of renal cortical tissues. The signal intensity of Oat3 in membrane and whole cell lysate fractions normalized to β-actin. Bar graphs indicate mean ± SEM (from 6 rats in each group). *P < 0.05 compared to the control group, #P < 0.05 compared to the gentamicin-treated group.
60x81mm (300 x 300 DPI)

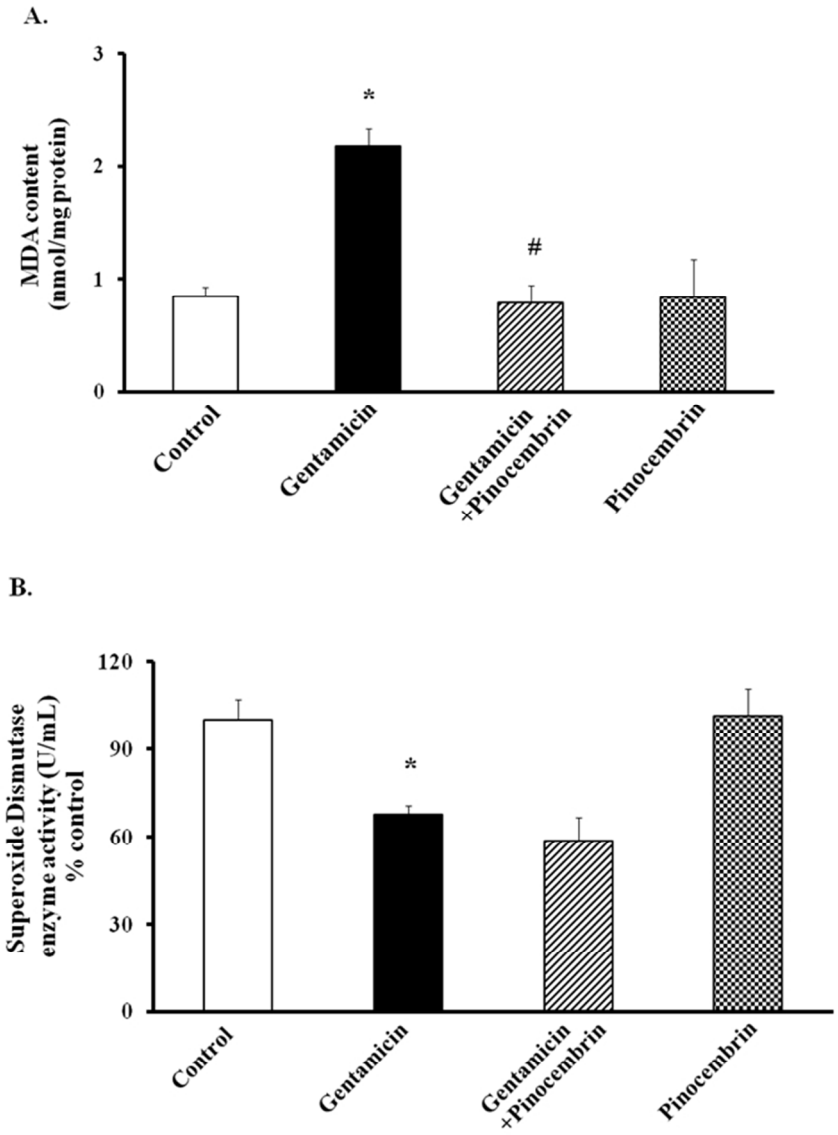

Figure 3 A. Effects of pinocembrin pretreatment on the renal cortical MDA concentration. Thiobarbituric acid reactive substances (TBARS) were measured in renal cortical tissues. B. Effects of pinocembrin pretreatment on SOD enzyme activity in renal cortical tissue. Values are the mean ± SEM. (n=6 rats in each group). *P < 0.05 compared to the control, #P < 0.05 compared to the gentamicin-treated group.
60x81mm (300 x 300 DPI)

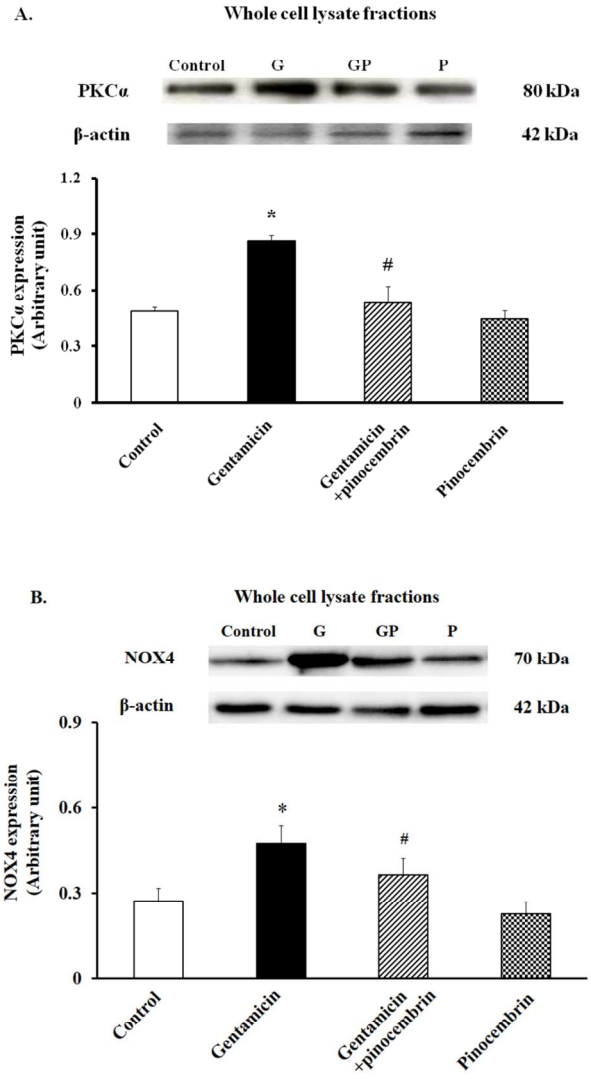

Figure 4 Effects of pinocembrin pretreatment on the expressions of PKCα (A) and NOX4 (B) in renal cortical tissues. Immunoblot analysis for PKCα and NOX4 expressions in whole cell lysate fraction of renal cortical tissue and immunostaining signal intensity of PKCα and NOX4 expressions normalized to β-actin. Bar graphs indicate mean ± SEM (n=6 rats in each group). *P< 0.05 compared to the control, #P< 0.05 compared to the gentamicin-treated group.
209x297mm (300 x 300 DPI)

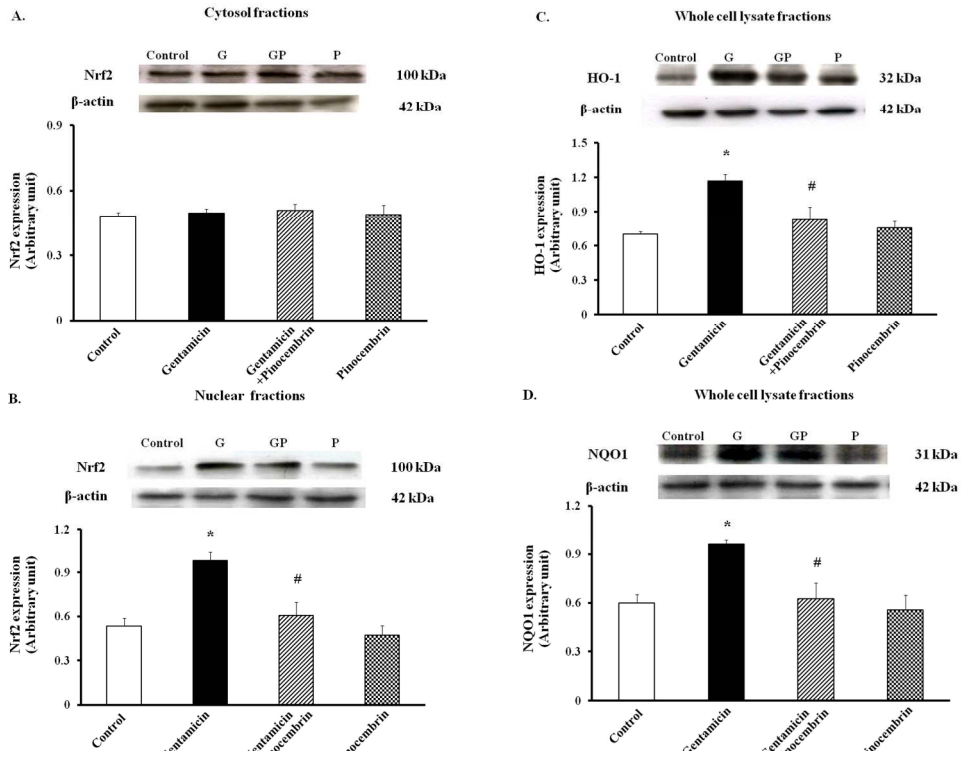

Figure 5 Effects of pinocembrin pretreatment on the expression of Nrf2 in the renal cortical tissue. Immunoblot analysis for Nrf2 in nuclear (A) Nrf2 in cytosolic fractions (B) HO-1 (C) and NQO1 (D) expressions of renal cortical tissues. Immunostaining signal intensity protein expressions normalized to β-actin. Bar graphs indicate mean ± SEM (n=6 rats in each group). *P < 0.05 compared to the control, #P < 0.05 compared to the gentamicin-treated group.
260x194mm (300 x 300 DPI)

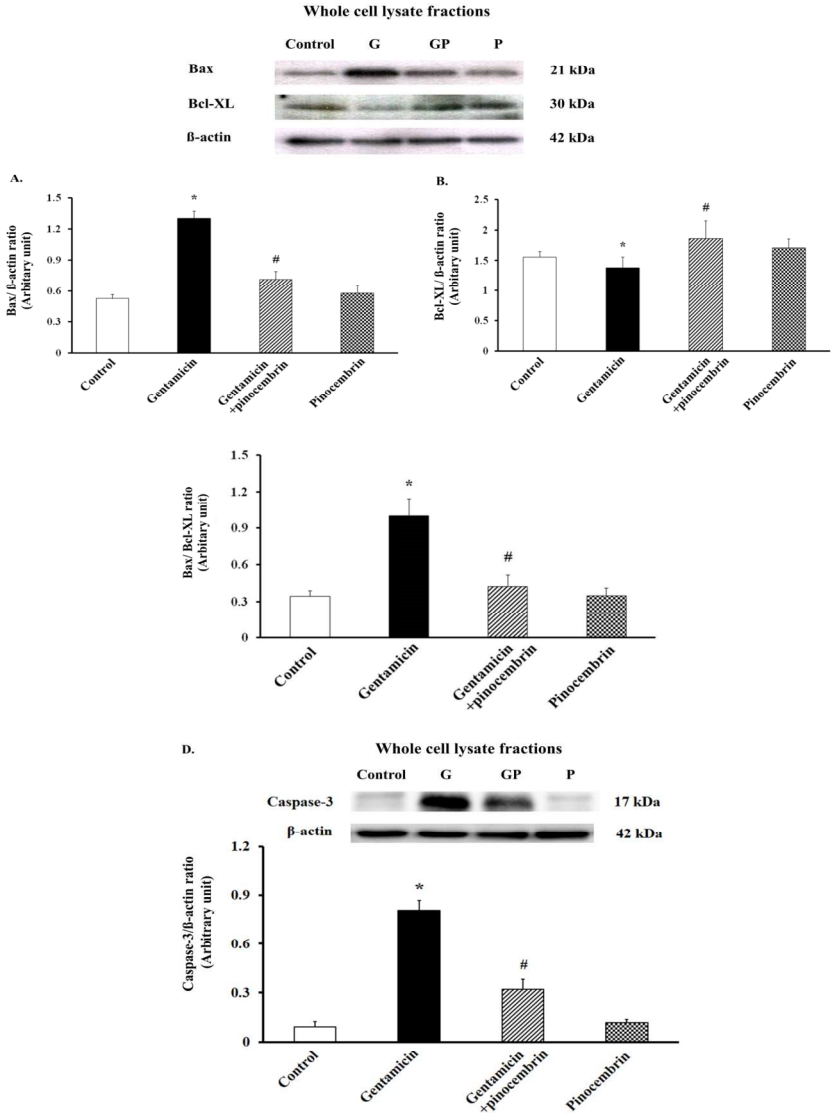

Figure 6 Effects of pinocembrin pretreatment on the expression of apoptotic proteins in renal cortical tissue. (A), (B) and (D): Representative immunoblot analysis for Bax, Bcl-XL and caspase-3 expressions in renal cortical tissues, respectively. (C): Immunostaining signal intensities of Bax/Bcl-XL ratio. Immunostaining signal intensities of Bax, Bcl-XL and caspase-3 expressions normalized to β-actin. Bar graphs indicate mean ± SEM (n=6 rats in each group). *P< 0.05 compared to the control, #P< 0.05 compared to the gentamicin-treated group.
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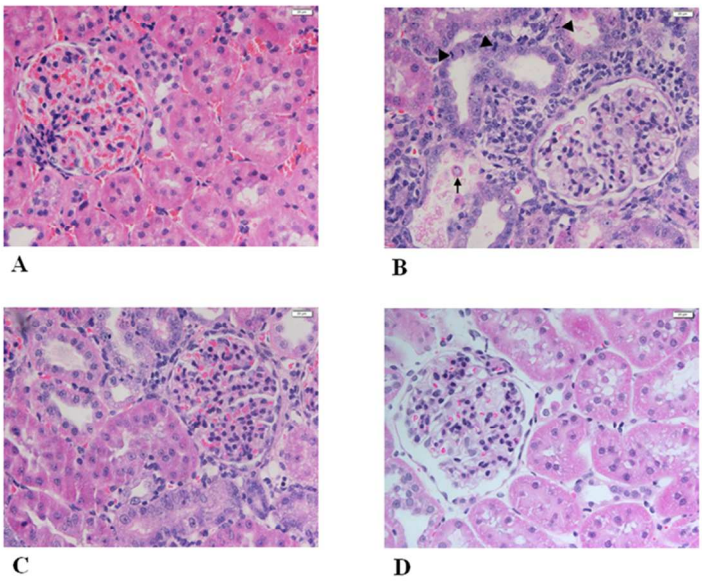

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420x297mm (300 x 300 DPI)