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## Protective effects of protocatechuic acid against cisplatin-induced renal damage in rats

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### ABSTRACT

The protective effects of an extract from bitter melon (*Momordica charantia*, Cucurbitaceae) against oxidative stress was previously reported and found that protocatechuic acid (PCA) was one of the major phenolic constituents in the extract. The renoprotective effect of PCA from bitter melon was investigated in the present study. In the LLC-PK1 cellular model, the decline in cells viabilities induced by oxidative stress, such as that induced by sodium nitroprusside, pyrogallol, and SIN-1, was significantly and dose-dependently inhibited by PCA. In the *in vivo* model, the cisplatin-treated rats showed increased plasma levels of creatinine, decreased creatinine clearance, and increased urine protein levels. However, these parameters related to renal dysfunction were markedly attenuated by PCA treatment. Administration of PCA resulted in remarkable improvement in the histological appearance and reduction in tubular cell damage in the cisplatin-treated rat kidneys. Moreover, the elevated levels of pro-caspase-3 induced by cisplatin in rat kidneys were down-regulated by PCA co-treatment. These results suggest that PCA has protective activity against anticancer drug-induced oxidative nephrotoxicity.

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## 1. Introduction

Bitter melon (*Momordica charantia*) is an important cultivated food crop widely consumed in Asia. It has been reported to have various medicinal effects, such as hypoglycaemic, anti-rheumatic, anti-inflammatory, anticarcinogenic, hypocholesterolaemic, antiseptic, and anti-diabetic (Anila & Vijayalakshmi, 2000; Leatherdale et al., 1981; Lotikar & Rajarama Rao, 1966). The main constituents exhibiting these effects have known to be triterpenes, proteids, steroids, alkaloids, inorganics, lipids, and phenolic compounds (Choi, Kim, Seo, Lee, & Cho, 2012).

We previously reported the protective effects of methanol extract against oxidative stress. Among fractions obtained from the extract with different organic solvents, the butanol fraction had the strongest activity (Sin et al., 2011). Protocatechuic acid (3,4-dihydroxybenzoic acid, PCA) was one of the most abundant phenolic compounds in the extract (Choi et al., 2012). PCA has been found in many food plants including olives, *Hibiscus sabdariffa*, and *Eucommia ulmoides* (Lin et al., 2003; Tsai & Yin, 2012). The biological activities of this compound, such as antioxidant, antibacterial, anticancer, antiulcer, antidiabetic, antiageing, antifibrotic, antiviral, anti-inflammatory, analgesic, antiatherosclerotic, cardiac, hepatoprotective, neurological, and renoprotective activities have been reported (Rao & Liang, 1980; Rao, Liu, Gao, Liang, & Zhu, 1988; Shi, An, Jiang, Guan, & Bao, 2006; Tanaka, Tanaka, & Tanaka, 2011).

Cisplatin (cis-diamminedichloroplatinum II) is one of the most potent antitumour drugs used against a wide spectrum of malignancies; however, its application for counteracting cancer is restricted due to the development of nephrotoxicity. Despite intensive care measures, one-third of cisplatin-treated patients experience irreversible renal damage (Taguchi, Nazneen, Abid, & Razzaque, 2005).

Natural phenolics, such as hesperidin, rutin, silymarin, and genistein, have been shown to ameliorate cisplatin-mediated nephrotoxicity (Kang et al., 2011; Sahu, Kuncha, Sindhura, & Sistla, 2013). Moreover, quercetin prevented the nephrotoxic activity of cisplatin without affecting its anti-tumour activity (Sanchez-Gonzalez, Lopez-Hernandez, Perez-Barriocanal, Morales, & Lopez-Novoa, 2011). These findings indicate that natural phenolic compounds could be used as nephroprotective agents for ameliorating cisplatin-induced kidney injury.

Antioxidants that prevent cellular damage induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered to be effective therapeutic agents for kidney diseases (Djamali, 2007; Naqshbandi, Rizwan, & Khan, 2013; Verzola et al., 2004). Therefore, to elucidate the renoprotective effect of protocatechuic acid, we investigated the protective effects of protocatechuic acid against cisplatin-induced oxidative stress in cultured kidney cells and in rats.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Sodium nitroprusside (SNP), a metabolic generator of NO, was purchased from Wako Pure Chemical Industries Ltd. (Richmond,

VA). 3-Morpholinopyridone (SIN-1), pyrogallol, protocatechuic acid (PCA, purity: 98–100%), cisplatin (purity: 98–100%), tetrahydrocurcumin (THC, purity: 98–100%) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma Chemical (Perth, WA). LLC-PK1 porcine renal epithelial cells were provided by ATCC. Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). Stock solutions of chemicals were prepared in 100% dimethylsulphoxide (DMSO), and stored at  $-20^{\circ}\text{C}$  until used. When required, the stock solution was diluted with cell culture media to the appropriate concentration. The final concentrations of DMSO were adjusted to less than 0.5% (v/v) in the culture media. Organic cation transporter 2 (OCT-2), procaspase 3 and GAPDH conjugated anti-rabbit antibodies were purchased from Cell Signaling Technology (Danvers, MA).

### 2.2. Cell cultures

Commercially available LLC-PK1 cells were maintained in culture flasks containing 5% FBS-supplemented DMEM/F-12 medium (pH 7.2) at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  in air. All subsequent procedures were carried out under these conditions. The cells were sub-cultured 5 days with 0.05% trypsin-ethylenediaminetetra acetic acid (EDTA) in phosphate-buffered saline (PBS).

A generator-induced cellular oxidative model was employed to investigate the protective effects of protocatechuic acid from oxidative damage (Yokozawa, Rhyu, & Cho, 2003). After confluence had been reached, the cells were plated into 96-well plates at  $10^4$  cells/ml and allowed to adhere for 2 h. SNP (1.2 mM), pyrogallol (0.25 mM) and SIN-1 (1.0 mM) were treated to generate nitric oxide (NO), superoxide ( $\text{O}_2^-$ ) and peroxynitrite ( $\text{ONOO}^-$ ), respectively. After 24 h of incubation, protocatechuic acid were treated in the test wells at various concentrations for 2 h.

Cell viability was assessed using the MTT colorimetric assay. MTT solution (1 mg/ml) was added to each 96-well culture plate and incubated for 4 h at  $37^{\circ}\text{C}$  and then the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100  $\mu\text{l}$  dimethyl sulfoxide (DMSO) and the absorbance at 540 nm of each well was read using a microplate reader (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987).

### 2.3. Animals

All procedures involving the use of live animals as described in this study were approved at May 2014 meeting of the Institutional Animal Care and Use Committee of Gachon University (approval number: GIACUC-R2014002) and strictly followed the NIH guidelines for humane treatment of animals. Male Wistar rats weighing 140–160 g were used for evaluating the protection of the PCA against cisplatin-induced nephrotoxicity. The rats were housed at  $23 \pm 2^{\circ}\text{C}$ ,  $55 \pm 5\%$  humidity, with a standard cycle of 12 h light/dark. The rats were given free access to water and normal diet containing 10% fat for a period of one week after arrival.

## 2.4. Experimental design

The animals were divided into four groups (four rats in each group):

- Group I: Vehicle (n = 4) received water (no sample treatment).
- Group II: Cisplatin (n = 4) received water (no sample treatment).
- Group III: Cisplatin + PCA (n = 4) treated with PCA (10 mg/kg) in aqueous solution orally for 10 days.
- Group IV: Cisplatin + PCA (n = 4) treated with PCA (20 mg/kg) in aqueous solution orally for 10 days.
- Group V: Cisplatin + THC (n = 4) treated with THC (20 mg/kg) in aqueous solution orally for 10 days.

PCA was orally administered every day at a dose of 10 and 20 mg/kg body weight, while vehicle-treated rats were orally given water. The PCA dosage was determined on the basis of previous studies (Murugan & Pari, 2006). THC was used as the positive control. After 4 days, the rats in two groups (cisplatin and cisplatin + PCA) were administered a single dose of cisplatin intraperitoneally (7.5 mg/kg body weight) in 0.9% saline. Animals in the vehicle group received an equivalent amount of normal saline for 10 days. The rats were sacrificed 6 days after cisplatin administration under light ether anaesthesia. The urine samples (24 h) were collected using metabolic cages. Blood samples were collected from abdominal aorta and kidneys were removed. All the preparations and analyses of various parameters were performed simultaneously under similar experimental conditions to avoid any day to day variations.

During the experimental period, the rats' body weights were measured daily.

## 2.5. Plasma biomarker analyses

Blood samples were collected in tubes containing 0.18 M EDTA and centrifuged at 3000 g for 5 min at 4 °C. After centrifugation, plasma was separated for estimation of total cholesterol and creatinine. Creatinine levels were determined by a rate-blanked kinetic Jaffe method. Creatinine clearance was calculated on the basis of the urinary Cr, serum Cr, urine volume, and body weight using the following equation: Creatinine clearance (ml/kg body weight/min) = [urinary Cr (mg/dl) × urine volume (ml)/serum Cr (mg/dl)] × [1000/body weight (g)] × [1/1440 (min)].

## 2.6. Histological examination

Kidney sections were fixed in 10% neutral-buffered formalin solution for 48 hours, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin wax blocks. Three to five µm sections were cut using a Leica microtome, washed in a water bath and left in the oven for dewaxing. The sections were stained with haematoxylin and eosin (H&E) and Masson's trichrome (MTC), as reported previously (Bancroft & Gamble, 2002). The H&E stain revealed the general histological architecture of the renal parenchyma. MTC stain was used for demonstration of the collagen connective tissue distribution within the renal structure. The stained tissue slides were

covered with cover slips to be examined under a light microscope.

## 2.7. Western blot

The kidney tissues were homogenized in a protein extraction solution, after which the homogenates were centrifuged (12,000 × g, 15 min, 4 °C), and the protein concentrations in the supernatants were determined using the Bradford protein microassay (Bio-Rad, Hercules, CA, USA). The centrifuged samples were mixed with the same volume of 2 × SDS sample buffer [62.5 mM, Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 125mMDTT, 0.3% (w/v) bromophenol blue], to give a final buffer concentration of 1 × (500 µg/100 µl). The homogenates were boiled at 94 °C for 5 min in Laemmli sample buffer. Total protein (40 µg) were then loaded onto a stacking polyacrylamide gel and resolved in an 8–15% polyacrylamide gel, along with biotinylated molecular-weight standard markers. The separated proteins were wet-transferred to a 0.2-µm nitrocellulose membrane. Subsequently, the blots were blocked for 1 h with 5% nonfat dry milk in TBST buffer [20 mM Tris-HCl (pH 7.6), 0.8% NaCl, 0.05% Tween 20] and incubated overnight at 4 °C with a 1:1000 dilution of antibody to procaspase-3, OCT-2 and GAPDH. After washing in TBST, the blots were incubated with HRP-conjugated secondary antibody (1:2000 dilution, anti-rabbit) for 1 h at room temperature, washed three times, and then detected with ECL solution.

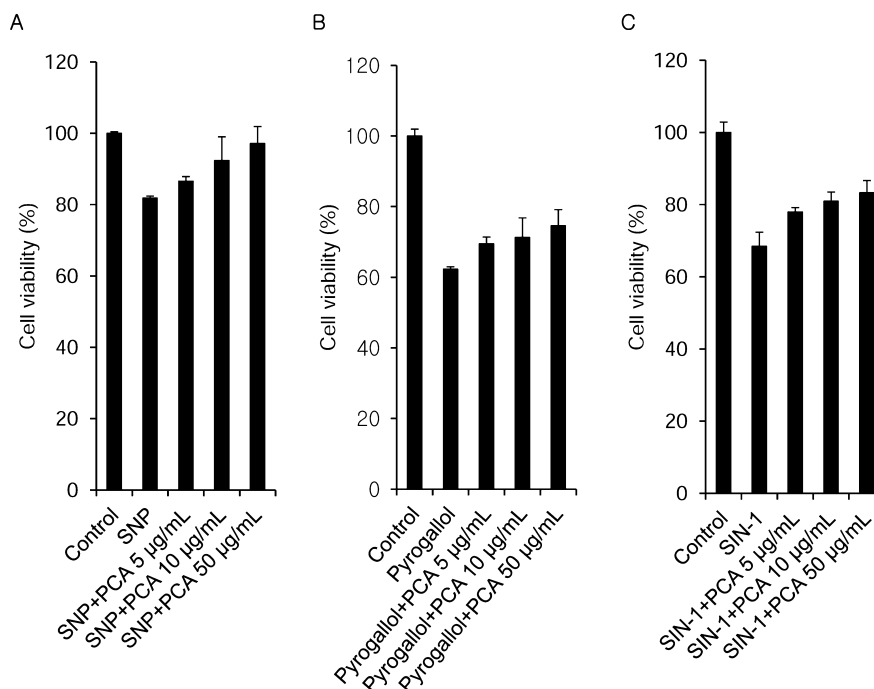
## 2.8. Statistical analysis

All data were expressed as mean ± SD, then subjected to statistical analysis using SPSS software version 16. The significance of differences between the mean values was calculated using unpaired Student's t-test. P-value < 0.05 was considered to be statistically significant.

# 3. Results and discussion

The protective effects of PCA against NO-induced decrease in cell viability in a cellular system are shown in Fig. 1. Cell viability markedly decreased, falling to 81.9% due to the generation of NO by SNP, compared to that of cells that were not treated with SNP. PCA, however, caused a dose-dependent increase in cell viability. Cell viability increased to 92.4% and 97.2% when cells were treated with 10 and 50 µg/ml of PCA, respectively, after NO generation (Fig. 1A). As shown in Fig. 1B, O<sub>2</sub><sup>-</sup> generated by pyrogallol decreased the cell viability to 62.3% compared to 100% viability in the non-treated cells. When the cells were treated with PCA at concentrations of 5, 10, and 50 µg/ml, cell viability increased to 69.5%, 71.3%, and 74.6%, respectively. Also, cell viability declined to 68.5% after treatment with SIN-1, and treatment with PCA increased the cell viability in a dose-dependent manner (Fig. 1C). In particular, at a concentration of 50 µg/ml, cell viability increased to more than 83%.

Acute nephrotoxicity was evident by renal function impairment in the cisplatin-treated animals. On day 4 post-injection, cisplatin-treated rats showed increased plasma levels of



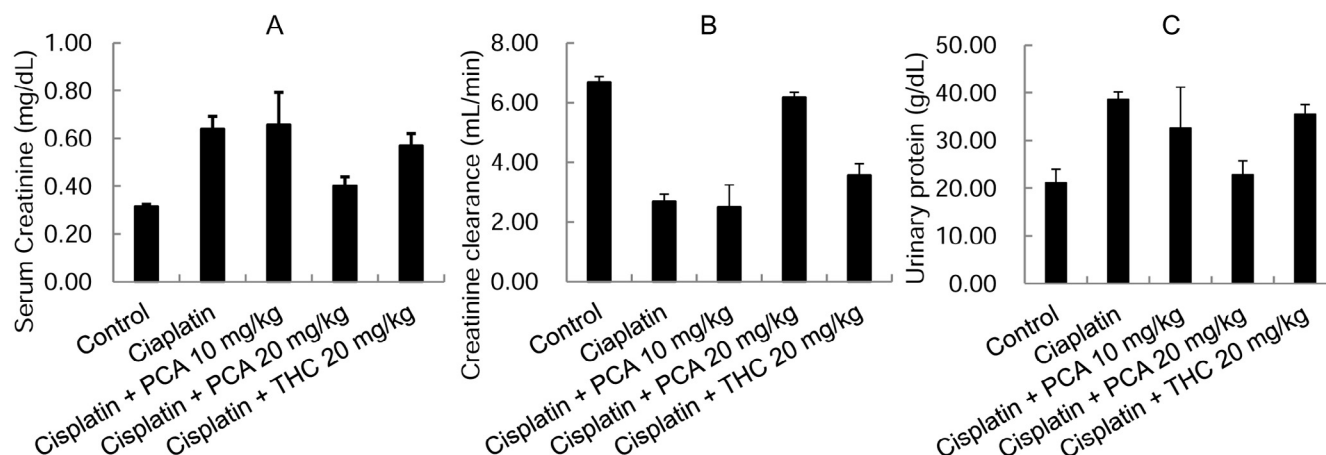
**Fig. 1 – Effects of protocatechuic acid on oxidative stress-induced renal cell damage. (A) Effects of protocatechuic acid on nitric oxide-induced renal cell damage. (B) Effects of protocatechuic acid on superoxide-induced renal cell damage. (C) Effects of protocatechuic acid on peroxynitrite-induced renal cell damage.**

creatinine and decreased creatinine clearance; these changes were prevented by PCA administration and their effect was stronger than that of THC (Fig. 2A and B). In addition, cisplatin-induced increase in the urine protein level was attenuated by PCA treatment (Fig. 2C).

Fig. 3 shows the protein expressions of procaspase-3 and OCT-2 in the control and experimental groups of rats. OCT-2 protein expression in the kidney tissue increased slightly after cisplatin treatment and decreased after PCA treatment, but the differences were not statistically significant (Fig. 3B). Procaspase-3 protein expression levels increased significantly

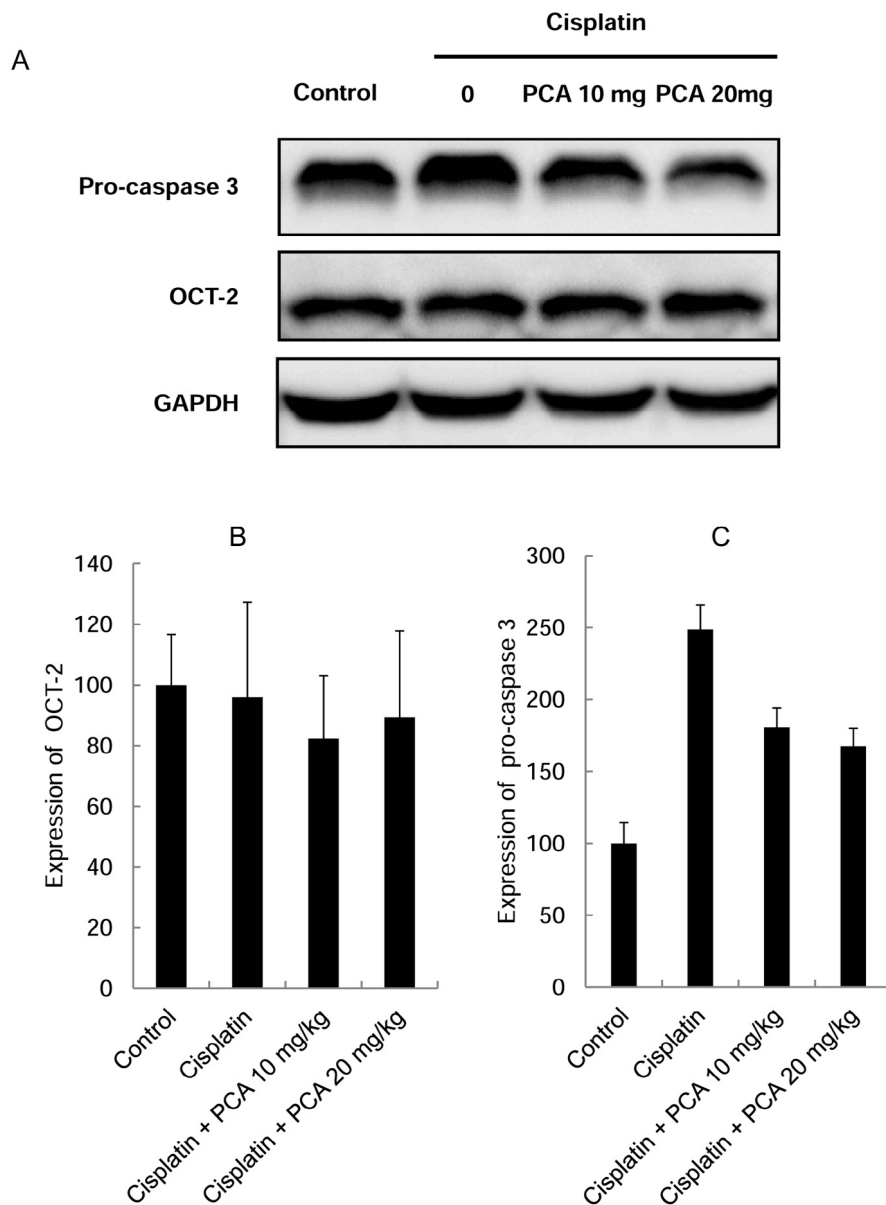
after cisplatin injection, but co-treatment with PCA resulted in almost complete renoprotection (Fig. 3C).

Fig. 4 shows the histopathological changes in the rat kidney stained with H&E and viewed at the original magnification of  $\times 100$ . Images were selected from the glomerular, tubular sections: normal control rats showed normal architecture of the kidney (Fig. 4A). Extensive tubular epithelial cellular necrosis, desquamation, vacuolization, and swelling were observed in the cisplatin-treated group, as shown in Fig. 4B. Administration of PCA resulted in remarkable improvement in the histological appearance, and in the reduction in tubular



**Fig. 2 – Effects of PCA on serum creatinine, renal function parameter and urinary protein in the cisplatin-induced renal damage rat model. (A) Serum creatinine. (B) Creatinine clearance. (C) Urinary protein.  $p < 0.05$  compared to the cisplatin-treated control value. Tetrahydrocurcumin was used as the positive control.**





**Fig. 3 – Effects of PCA on the protein expression levels of pro-caspase-3 and OCT-2 of rat kidneys. (A) OCT-2 and procaspase-3 protein expressions evaluated by Western blot analysis. (B) Quantitative data for the OCT-2 Western blot analysis of renal cortex tissues. (C) Quantitative data for the procaspase-3 Western blot analysis of renal cortex tissues.  $p < 0.05$  compared to the cisplatin-treated control value.**

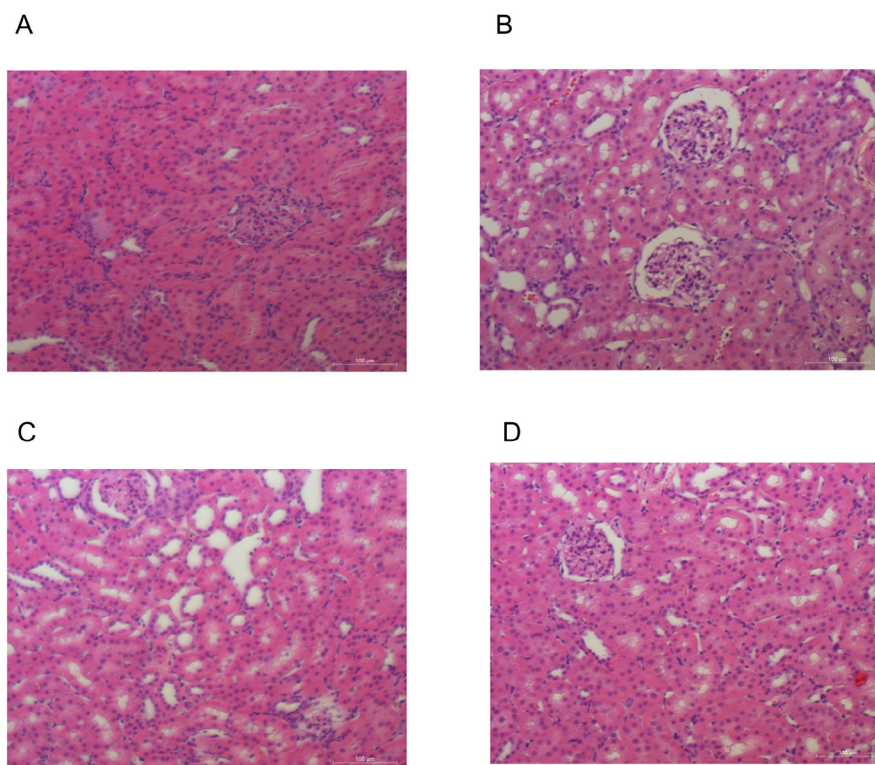
cell damage, swelling, and other changes as seen in Fig. 4C and D.

The control rats showed normal distribution of collagen fibres in the capsular wall, peritubular matrix, and around the blood vessels within the renal cortex (Fig. 5A). The amount and distribution of the collagen fibres showed recovery of the morphology in the PCA-treated rats compared to the cisplatin-treated rats (Fig. 5B–D).

Increased oxidative stresses mediated by ROSs have been implicated in a variety of kidney diseases (Djamali, 2007; Verzola et al., 2004). ROSs can be generated within the nephron segments like the glomeruli and the proximal tubule, and injury initiated by the lack of oxygen during cold presentation of renal transplantation is augmented by ROSs during subsequent warm

reperfusion of grafts through activation of the inflammatory cascade (Kaushal, Singh, & Shah, 1988). Also, ROSs play a critical role in the pathogenesis of cisplatin-induced nephrotoxicity. ROSs-induced cell death has been reported in renal proximal tubular epithelial cells (RPTECs), and ROSs also promote cisplatin-induced renal failure. ROSs directly damage the cell components, including lipids, proteins, and DNA, and activates the mitochondrial pathway of apoptosis, thus highlighting the interactions between both the processes (Choi et al., 2009; Evans, Wilson, & Guthrie, 2014).

The aim of the present study was to investigate the renoprotective effect of protocatechuic acid from bitter melon. LLC-PK1 renal epithelial cells are known to be susceptible to oxidative stress (Yao, Panichpisal, Kurtzman, & Nuqent, 2007).



**Fig. 4 – Effects of PCA on histopathological analysis of rat kidneys stained with H&E viewed at original magnification (100×). Images were selected from the glomerular, tubular sections. (A) Control rats. (B) Cisplatin-treated rats. (C) Cisplatin and 10 mg PCA-treated rats. (D) Cisplatin and 20 mg PCA-treated rats.**

A generator-induced cellular oxidative model was employed to investigate the protective effects of protocatechuic acid against oxidative damage. In the present study, the oxidative damage in LLC-PK1 cells exposed to free radicals such as NO,  $O_2^-$ , and  $ONOO^-$  was significantly and dose-dependently inhibited by protocatechuic acid co-treatment. Regarding the *in vivo* investigation of the renoprotective effect of PCA, the cisplatin-treated rats showed increased plasma levels of creatinine, decreased creatinine clearance, and increased urine protein levels. However, these parameters related to renal dysfunction were markedly attenuated by PCA treatment. Administration of PCA resulted in remarkable improvement in the histological appearance and reduction in tubular cell damage in the cisplatin-treated rat kidneys. In addition, the amount and distribution of the collagen fibres showed recovery of the morphology in the PCA-treated rats compared to the cisplatin-treated rats.

ROSs play an important role in the mediation of apoptosis by inducing the activation of caspases. Among all of the caspase members, caspase-3 in particular is an essential apoptotic effector that leads to cytoskeletal breakdown, nuclear, and other cell changes associated with apoptosis (Bratton & Cohen, 2001; Chen, Chien, Huang, & Chia, 2014). Mechanisms that activate caspase-8 and caspase-9 or executioner caspase-3 are known to be involved in cisplatin-induced tubular cell apoptosis, and caspase inhibition markedly reduces kidney injury (Jo, Cho, Sung, Kim, & Won, 2005). The present study showed that the elevated expression of pro-caspase-3 induced by cisplatin in the rat kidneys was down-regulated by treatment with

protocatechuic acid. These results suggest that protocatechuic acid alleviates cisplatin-induced oxidative renal damage by inhibiting the expression of pro-caspase-3.

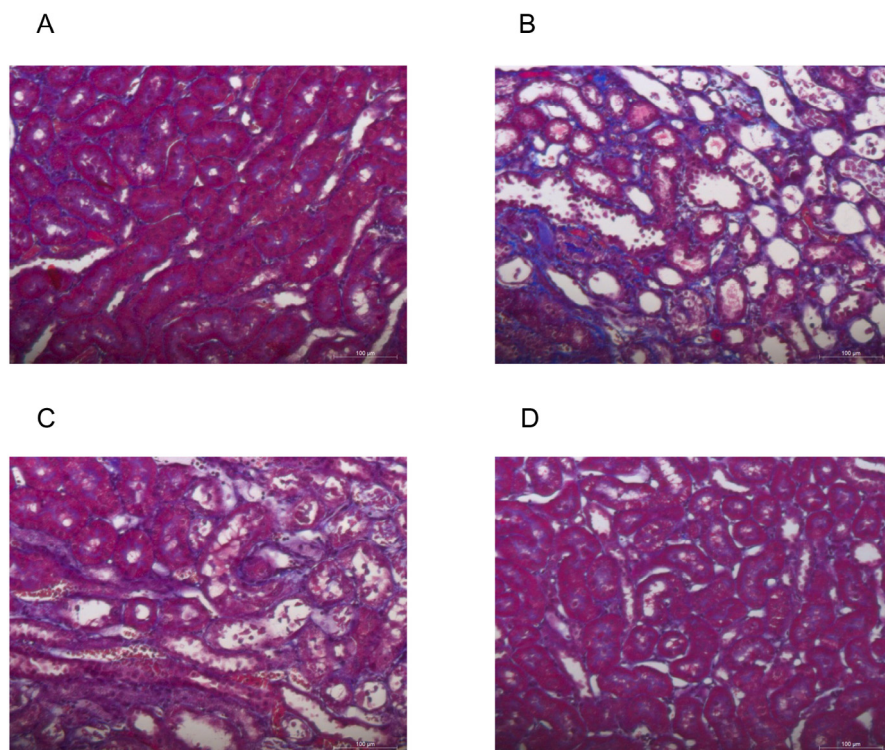
In summary, we demonstrated that PCA from bitter melon showed a protective effect against cisplatin-induced nephrotoxicity in cultured kidney cells and in rats. In the LLC-PK1 cellular model, reduced cell viabilities induced by oxidative stress such as that induced by NO,  $O_2^-$ , and  $ONOO^-$  were significantly and dose-dependently inhibited by PCA co-treatment. In addition, PCA attenuated the renal dysfunction induced by cisplatin via down-regulation of pro-caspase-3 expression in the kidney. These results indicate that PCA may have protective activity against anticancer drug-induced oxidative nephrotoxicity.

### Conflict of interest

The authors declare no conflict of interest.

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**Fig. 5 – Effects of PCA on histopathological analysis of rat kidney stained with MTC viewed at original magnification (100×). Images were selected from the glomerular, tubular sections. (A) Control rats. (B) Cisplatin-treated rats. (C) Cisplatin and 10 mg PCA-treated rats. (D) Cisplatin and 20 mg PCA-treated rats.**

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