Full Length Research Paper

Curcuma comosa ameliorates cisplatin-induced nephrotoxicity: COX-2 expression and ultrastructure changes

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To determine the protective effects of ethanol extracts of *Curcuma comosa* on kidney injury by cisplatin, mice were randomly assigned into 4 groups: Control, cisplatin control (12.5 mg/kg body weight (BW), i.p.), *C. comosa*+cisplatin (pretreatment with *C. comosa* at dose 200 mg/kg BW orally for 4 consecutive days before cisplatin injection), and *C. comosa* control groups. After five days, the renal tissues were collected to evaluate histopathological changes and inflammatory markers. This study elucidates the postulate of using *C. comosa* to counteract effect of cisplatin in terms of renal toxicity. The outcome shows that incidence of nephrotoxicity in cisplatin given along with *C.comosa*, decreased clinically and statistically, comparing with cisplatin given alone. It shows less of renal tubular damages and COX-2 expression. Also, the microscopic alteration showed a decreased number of swollen cells, necrotic and apoptotic cells. These aforementioned results proved the benefit use of *C. comosa* in aspect of renal protection. *C. comosa* can ameliorate cisplatin-induced kidney injury through the suppression of the inflammatory cytokine (COX-2) and its anti-oxidant properties. Therefore, it is a promising alternative regimen for the prevention of nephrotoxicity during cisplatin therapy.

Key words: Randomized controlled trial, *Curcuma comosa*, kidney injury prevention, histopathology, COX-2 expression.

INTRODUCTION

cis-Dichlorodiammine platinum (II) or Cisplatin is one of the most effective alkylating agent widespread use in chemotherapy regimen for solid organ tumor including head, neck, lung, testis, ovary and breast. As a platinum-based anticancer agent, cisplatin acts through inhibition of DNA inter-strand and intra-strand cross-linking process. This mechanism leads to decreased cell proliferation; and eventually cell death (Chvalova et al., 2007). Despites the use of cancer eradication, cisplatin also causes many harmful side effects such as, ototoxicity, gastro-toxicity, myelosuppression and nephrotoxicity, with the incidence up to 20% in those

receiving high-dosed cisplatin (Hill and Speer, 1982; Cooley et al., 1994). The pathogenesis of cisplatin-induced kidney injury is caused by direct cell injury from the uptake of free-form cisplatin in tubular cells. This uptake can be observed at inner medullae and outer cortices as well. As a result, this substance caused tubular dysfunction and lead to kidney injury (Kuhlmann et al., 1997).

Damage to kidney could be manifested in many ways such as reduction of glomerular filtration rate, reduction of renal blood flow, polyuria, hypomagnesemia, hypokalemia and hypocalcemia (Winston and Safirstein, 1985; Hutchison et al., 1988; Schilsky and Anderson, 1979). Many renal protective protocols have been postulated in order to lessen the occurring of cisplatin-induced nephrotoxicity, for example normal saline solutions, mannitol and furosemide infusion, and sodium thiosulphate (Cvitkovic et al., 1977; Pera et al., 1979; Heidemann et al., 1985; Hirosawa et al., 1989). According to ESRP SIG or European Society of Clinical Pharmacy Special Interest Group cancer care recommendation, only normal saline infusion was recommended to be used for the prevention of the adverse effect (Launay-Vacher et al., 2008). Even though aggressive hydration, especially with normal saline solutions as the suggestion is routinely performed, the number of nephrotoxicity is still not decreasing (Hayes et al., 1977; Einhorn and Donohue, 1977). Thus, the protective manner for cisplatin-induced nephrotoxicity is still needed for a new method.

Curcuma comosa Roxb. (*Curcuma* species, Zingiberaceae family) is commonly a traditional herbal medicine under the name Wan-Chak-Mod-Luk in Thailand. Thai traditional practitioners use its rhizome as an anti-inflammatory agent for the treatment of postpartum uterine bleeding. It enhanced involution and reduced inflammation of uterus after vaginal delivery (Piyachaturawat et al., 1995). Interestingly, *C. comosa* and its diarylheptanoid component were reported to have anti-oxidant and anti-inflammatory properties (Jantaranotai et al., 2006; Suksamrarn et al., 2008). Its anti-inflammatory effect is proved by the evidence of reduction in release of pro-inflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin-1β from monocytoid U937 cell line in phorbol-12-myristate-13-acetate (PMA)-stimulated PBMC. The two *C. comosa* diarylhepatanoids were mentioned to reduce the expression of TNF-α and suppress expression of IκB kinase and activation of nuclear factor kappa B (Sodsai et al., 2007). Moreover, the ethanol extract of *C. comosa* exhibited effective protection against cisplatin-induced nephrotoxicity through its antioxidant activity by exhibiting radical scavenging activities, such as lipid peroxidation, glutathione (GSH) content, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities (Jariyawat et al., 2009). Thus, this study was intended to investigate the ameliorative effects of ethanol extract of *C. comosa* on cisplatin-induced nephrotoxicity in mice for the possibility of usage as a pretreatment regimen in the future.

MATERIALS AND METHODS

Ethanol extract of *C. comosa* and chemicals

The rhizomes of *C. comosa* were sliced, dried at 50 to 60°C, pulverized and extracted in a Soxhlet extraction apparatus with hexane first and then with ethanol. The *C. comosa* ethanol extract was dissolved in 10% dimethyl sulfoxide (DMSO) and suspended in olive oil later. The two main substances extracted are 7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol and (3S)-7-(3, 4-dihydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol. All chemicals and solvents used throughout this investigation were of analytical grade. Cisplatin was purchased from Pharmacia (Perth) Pty limited (WA, Australia) and dissolved in saline to give a 1 mg/ml solution.

Animal and experimental design

Male ICR mice (8 weeks old, 25 to 30 g) were obtained from the National Animal Center of Thailand, Mahidol University, Salaya Campus, Nakornpathom, Thailand. They were given a standard laboratory diet and water *ad libitum,* maintained in room with controlled temperature (25 ± 2°C), humidity 65% and a 12-h dark/light cycle, and allowed to acclimatize for 1 week before use. All experimental mice were performed in accordance with the guidelines of National Laboratory Animal Center, Mahidol University, Bangkok, Thailand.

Forty mice were randomly assigned into 4 groups of 10 animals each: solvent control group, Cisplatin group (12.5 mg/kg body weight (BW), intra-peritoneal or i.p.), *C. comosa*+cisplatin group (pretreatment with *C. comosa* extract at dose 200 mg/kg BW orally for 4 consecutive days before cisplatin injection), and *C. comosa* group. Five days after kidney removal in all mice, unfixed cryostat sections (6 μm thick) were prepared for cyclooxygenase-2 or COX-2 staining. The renal tissues were then collected and fixed in 4% neutral buffered paraformaldehyde for evaluation under light microscopy (LM) and 2.5% glutaraldehyde for evaluation under transmission electron microscopy (EM). The project was submitted and approved by the Siriraj Animal Care and Use Committee (SI-ACUC), Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Histopathological preparation

Light microscopy

The mouse kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, cross-sectioned (4-μm thickness), and stained with hematoxylin-eosin. The histopathological analyses were performed blindly under a light microscope (LM). The renal sections graded by semi-quantitative scale to evaluate the degree of tubular changes. The study of changes was limited to the area of proximal tubules, S1, S2 and S3. These parameters were evaluated under a 5-point scale: 0 = normal; 1 = minimal proximal tubular cells swelling; 2 = moderate proximal tubular cells swelling with cast in tubular lumen; 3= maximal proximal tubular cells swelling with cast in lumen, early necrosis and apoptosis of cells; 4 = necrosis, apoptosis and sloughing of proximal tubular cells. The mean score for each group was calculated. Statistical significance was assessed by the two-sided Student's t test for independent samples, and was indicated if

the *P*-value was 0.05 or less.

Transmission electron microscopy (TEM)

Upon animal sacrificing, renal samples were dissected into 1-mm cube tissue, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 1 to 2 h, rinsed with the buffer, post-fixed in 1% OsO_4 for 1 h, then rinsing again with the buffer and post-fix in 2% Uranyl acetate aqueous solution for 30 min. Then the specimens were rinsed in distilled water, dehydrated in a graded series of alcohols, treated with propylene oxide two times for 20 min each, immersed in the mixture of propylene oxide and araldite plastic (2:1) for 60 min and then immersed again in the mixture of propylene oxide and araldite plastic (1:2) for overnight, lastly embedded in araldite plastic, and overnight incubated. Ultrathin sections then were cut and mounted on the copper grids, washed in distilled water, viewed and photographed under transmission electron microscope.

Immuno-histochemistry cyclooxygenase staining

Fresh renal specimens were cut with cryostat at 4 µm thicknesses, air-dried and fixed in 0.1% formalin for 5 min. The sections were fixed in 95% ethanol for 20 min; and incubated with 3% H_2O_2 in ethanol for 5 min in order to inactivate endogenous peroxides. Non-specific antibody binding sites were locked by using 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) pH 7.4. The tissue sections were incubated in 1:200 rabbit polyclonal antibody to human cyclooxygenase-2 (COX-2) as primary solution in a humidified chamber at 4°C overnight, washed with PBS and then incubated for 1 h with peroxidase labeled polymer as secondary solution (1:1 Envision+/ HRP anti-rabbit antibody; Dako Laboratories), washed again with PBS and re-incubated in a solution of 0.1 M 3,3'-diaminobenzidine (DAB) in 0.05 M TBS with 0.5 ml 3% H_2O_2 DAB solution (Dako Laboratories) for 5 min. The slides were then counterstained with hematoxylin. The expression of COX-2 was stained in brownish color. The analysis of immuno-histochemical staining method was assessed by light microscopy and analyzed with four sections from each tissue block. The COX-2 staining was examined by one double blinded observer who was blinded to the origin of the sections. The expression of COX-2 was evaluated according to the intensity in semi-quantitative grading; 0=negative staining, 1= minimal, 2= mild, 3= moderated, 4= strong-positive staining. Images were captured at 400× magnification and the entire outer stripes of medulla were measured. Total immuno-stained (brown) cells were averaged and expressed as the mean of intensity of stained area per field on 20 microscopic fields, each responding to an area of 0.042 mm^2, mean score for each group was calculated.

Statistical analysis

Data are reported as the mean ± standard error of mean (SEM). Statistical different between groups were analyzed using one-way analysis of variance (ANOVA) and followed by Bonferroni test. Student's unpaired *t*-test was used for the evaluation of scores of renal damage between two groups. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Histopathological changes of renal tissues

The renal tissues of mice in control group (Figure 1A and E) and those given *C. comosa* (Figure 1 and H) showed no structural change in renal cortex and medulla. The renal tissues of mice induced by cisplatin revealed damages mainly at proximal tubular with widespread lesions to the outer stripe of outer medulla (S3 segment) and medullary ray of cortex. The affected proximal tubules displayed dilated lumens and large amount of necrotic epithelial cells, characterized by loss of brush border. Along with the aforementioned necrotic process, apoptosis was significantly noted as evidenced by diminished cell size with small amount of eosinophilic cytoplasm, condensed nuclear chromatin and karyorrhexis (Figure 1B and F). Comparing to cisplatin-given alone group, the renal tissues of those with *C. comosa*-pretreatment, showed positive improvement in the histopathology predominantly at proximal tubules in outer stripe of outer medulla (S3 segment) and medullary ray in cortex. Necrosis and apoptosis are noted less. The morphological change of this group showed only minimal cell swelling and few proximal tubular casts. The regeneration of the proximal tubular cells and lining epithelial cells, especially the S3 segment are also marked. The proximal tubular cells in this group are low cuboid-shaped cell with basophilic granular cytoplasm and large vesicular nuclei with numerous mitotic figures (Figure 1C and G). The descriptive and semi-quantitative grading of the structural change in each group is summarized in Table 1. The result reveals that higher score is significantly found in those with cisplatin-given alone comparing to those control group, *C. comosa*-given alone group, respectively (2.90 ± 0.10 vs. 0 vs. 0). On the other hand, score in *C. comosa* pretreatment with cisplatin treated group manifests markedly low in comparison to the group with cisplatin-given without *C. comosa* pretreatment (1.40 ± 0.16 vs. 2.90 ± 0.10).

Ultra-structural changes of renal tissues

Transmission electron microscopy (TEM) was used as a method to evaluate intracellular structural change in proximal tubular cells of kidney tissues from each group in this study. The tubular cells of cisplatin-given without *C. comosa*-pretreatment group showed typical apoptotic nucleus and early features of apoptosis, including cell shrinkage, nuclear chromatin clumping with accumulation at nuclear rim. Also, mitochondria were marked to be decreased in numbers; and differed in shape into wavy appearance with irregular or ruptured membrane. The lysosomes in this group were found to be in extremely electron-dense formations. The endoplasmic reticulums were also broken down into small vesicles. Most of the proximal cells showed stunt and expanded microvilli, and discontinuous plasma membrane (Figure 2B). Comparing to those cisplatin alone group, the severity of morphological damage of proximal tubules in the *C. comosa* pretreatment group was found to be less with evidence of decreased number of apoptotic cell (Figure 2C).
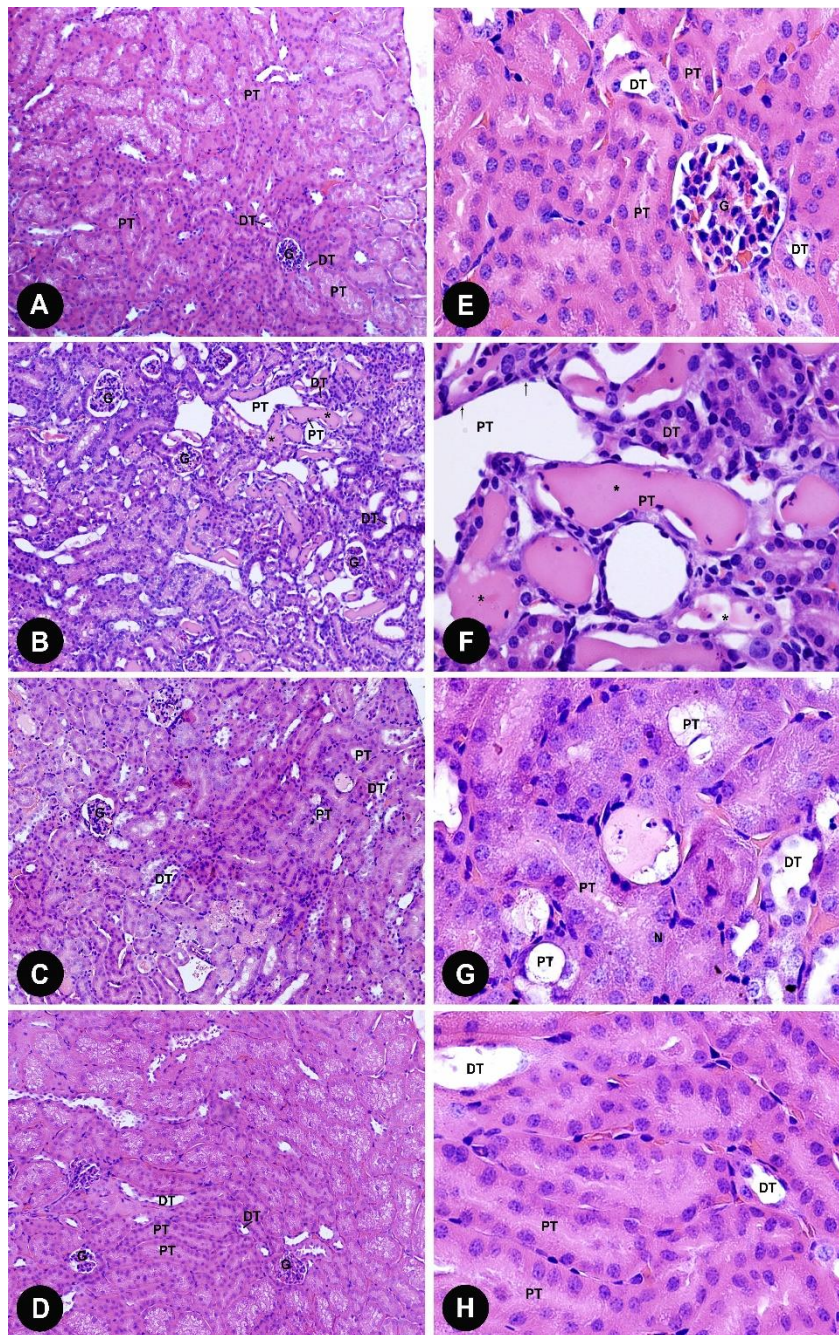
Figure 1. Light micrographs stained with H&E and the summary of semi-quantitative lesions after cisplatin administration, the extensive histological damages including tubular dilatation, cast formation (*), necrosis, apoptosis, loss of brush border and sloughing of proximal tubular cells (arrows) were observed in the cisplatin group (B, F). However, these changes were less pronounced in *C. comosa* pre-treated group **(**C, G**)**. Normal morphology of the proximal tubules was observed in control (A, E) and *C. comosa* control (D, H). The cisplatin-treated group revealed a significantly higher score versus controls. However, the pretreatment of *C. comosa* significantly lowered the score level compared with cisplatin treatment.

Cyclooxygenase-2 expression

COX-2 is an inducible enzyme which is usually undetectable in most normal tissues, unless during inflammation process. Five days after administration of cisplatin, COX-2 -positive cells were found in dark brown

Table 1. Descriptive and semi-quantitative grading of the structural change in each group.

Animal groups	n	Histopathological classification					Average score
		0	1	2	3	4	
Control	10	10	0	0	0	0	0.00 ± 0.00
Cisplatin	10	0	1	2	7	0	2.90 ± 0.10[a]
Cisplatin + *C. comosa*	10	3	6	1	0	0	1.40 ± 0.16[a,b]
C. comosa	10	10	0	0	0	0	0.00 ± 0.00

The level of tubular damages was scored at 5 levels; 0 = normal kidney; 1 = minimal proximal tubular cells swelling; 2 = moderate proximal tubular cells swelling with cast in tubular lumen; 3= maximal proximal tubular cells swelling with cast in lumen, early necrosis and apoptosis of cells; 4 = necrosis, apoptosis and sloughing of proximal tubular cells. Data are mean ± SE, n = number of mice. [a]$P<0.05$ versus control gr., [b]$P< 0.05$ versus cisplatin gr. (DT, distal tubule; G, glomerulus, PT, proximal tubule; A-D, x100; E-H, x400).
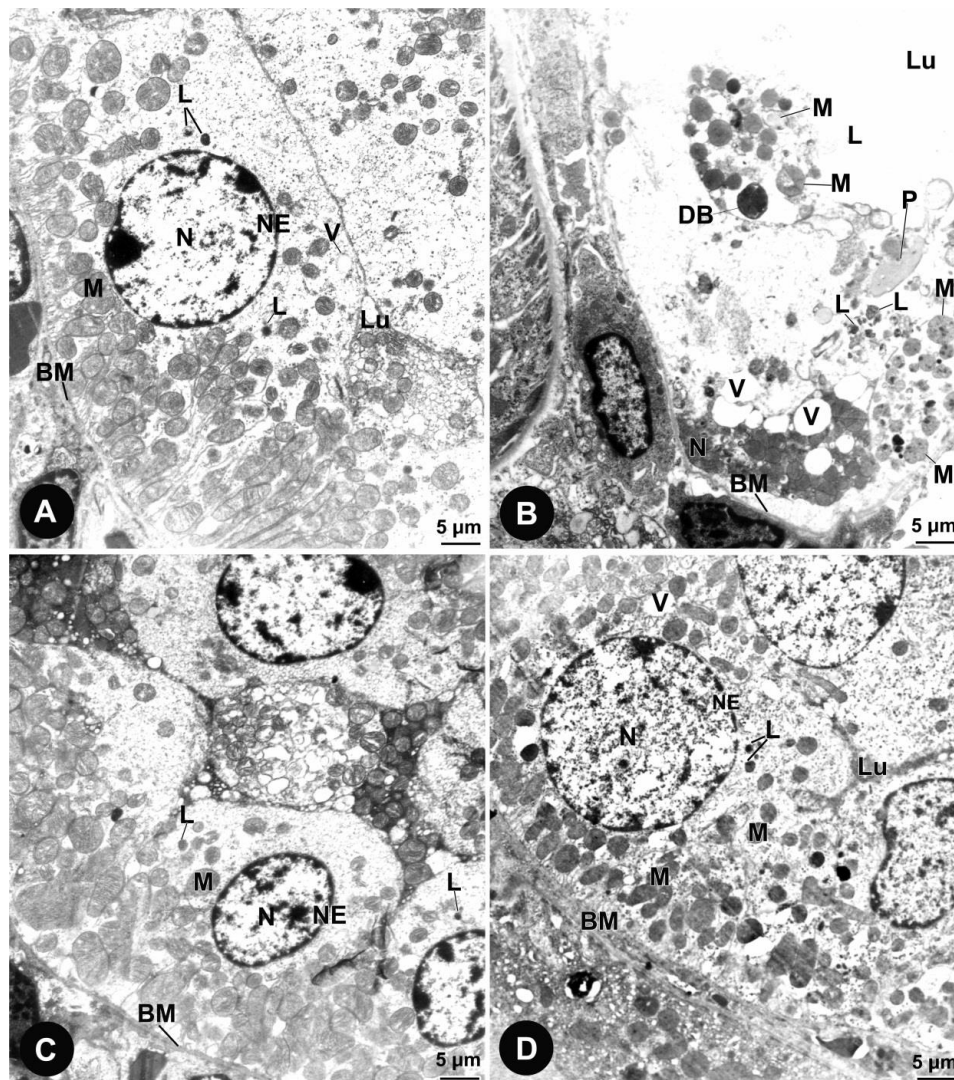

Figure 2. Transmission electron micrographs (TEMs) comparing part of proximal tubular cells of kidneys from various groups, apoptosis as evidenced by diminished cell sizes with condensed cytoplasm and chromatin, and loss of their brush border. The condensed chromosome accumulating at the nuclear rim and relatively intact cell organelles were observed in the cisplatin group (B). However, the tubular cell damage was reduced by pre-treatment with *C. comosa* (C) and normal tubular cells were observed in intact control (A) and *C. comosa* control (D) (DB, dense body; BM, basement membrane; L, lysosome; Lu, lumen; M, mitochondria; N, nucleus; NE, nuclear envelope; x3600).

color as peroxidase stain on the tissue sections. Accumulations of COX-2 expressed mainly at nuclear membrane, circumferential perinuclear cytoplasm, and diffuse cytoplasm (Figure 2B). The COX-2 expression was observed predominantly in the proximal tubules of outer stripes of outer medullas (S3 segment) and medullary rays. However, some parts in medullary rays were damaged beyond the inflammatory process into necrosis and apoptosis; whereas they expressed less staining. Comparing to control group, the COX-2 expression in cisplatin given alone group was significantly increased with mean score of 2.67 ± 0.37. The area involved mainly at the S3 segment of proximal tubules in the outer stripes of outer medullas (Figure 3). These changes were found to be in correlation with lesions found in histopathological and ultrastructural views.

With induction of *C. comosa* before cisplatin, the intense of COX-2 staining is profoundly decreased in the proximal tubules of the outer stripes of outer medullas and cortical medullary rays. The COX-2 expression is manifested in the same pattern as those cisplatin-given groups, but only in relatively small and scattered groups of cells. On immunohistochemistry detection, those with *C. comosa* pretreatment at dosage of 200 mg/kg showed marked decreased of COX-2 staining as compared to those of cisplatin alone group at the mean score of 1.32 ± 0.39 versus 2.67 ± 0.37 (Figure 3).

Thus, this study demonstrates the evidences of less inflammation process both in histopathology and immunohistochemistry aspects in pretreatment with *C. comosa,* in order to prevent nephrotoxicity in ciplatin given subjects.

DISCUSSION

Cisplatin has been chosen as a key drug to combat against various types of malignancies for decades. However, one third of the total numbers of patients are still suffering from the renal injury despite various pretreatment protocols (Prasaja et al., 2015). The prove of better outcome from *C. comosa* in this study will facilitate its novel use in standard practice.

In this study, we look into inflammatory cytokine COX-2 level and ultra-structural changes for evaluation of kidney injury. The outcome displays improvement of renal tissue in term of decreased pathologic changes, less tubular necrosis and apoptosis and reduction of expression levels of COX-2 evidently at proximal tubules in the outer stripes of outer medullas and cortical medullary rays in group with *C. comosa* pretreatment before cisplatin induction. Thus, *C. comosa* is proved in this study that it can be considered as a protective substance for cisplatin-induced nephrotoxicity. The mechanism of injury is caused by unbound free-form of cisplatin that filtered freely through glomeruli and uptake in tubular cells by various organic transporters. The concentration of the substance is highest at proximal tubular, followed by

inner medullae and outer cortices orderly (Kuhlmann et al., 1998). Once cisplatin is collected inside the cell, it causes damages by inducing reactive oxygen species which trigger program cell death or apoptosis through both intrinsic and extrinsic pathways (Ozben, 2007; Martindale and Holbrook, 2002). Besides, the oxidative stress injury directly to DNA. COX-2 expression is used to monitor inflammation markers in this study because of its ability to maintain in low level in baseline and elevated with cisplatin induction. Also, pathological changes are found to be correlated with area of high COX-2 expression (Jia et al., 2010). According to Jariyawat et al. (2009), the effect of free radical scavenger activities (EC_{50}) of diarylheptanoids extracted from ethanol extracts of *C.comosa*, 7-(4-hydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol and (3S)-7-(3,4-dihydroxyphenyl)-1-phenyl-(1E)-1-hepten-3-ol are 134.32 ± 7.14 and 6.14 ± 0.05 µg/ml, respectively. The ethanol extracts of *C. comosa* in this experiment has marked ability to counteract inflammation with EC_{50} 21.32 ± 0.40 µg/ml, vitamin C only has EC_{50} of 4.58 ± 0.18 µg/ml. The damaged area showing under LM, TEM correlates with the straining of COX-2. Thus, it is clear that the substance has direct effect of anti-oxidation process on kidney protection, however, the exact mechanism is sophisticated to be explained by one phenomenon.

In conclusion, this study exhibits convincing evidences in inflammation and morphological alterations, indicating benefit of *C. comosa* extract usage for renal protection through its effect of anti-inflammation and regeneration of tubular cells. Therefore, this extract could be a potential solution in the combination chemotherapy regimen with cisplatin to prevent from its kidney complication.

Conflict of Interests

The authors have not declared any conflict of interests.

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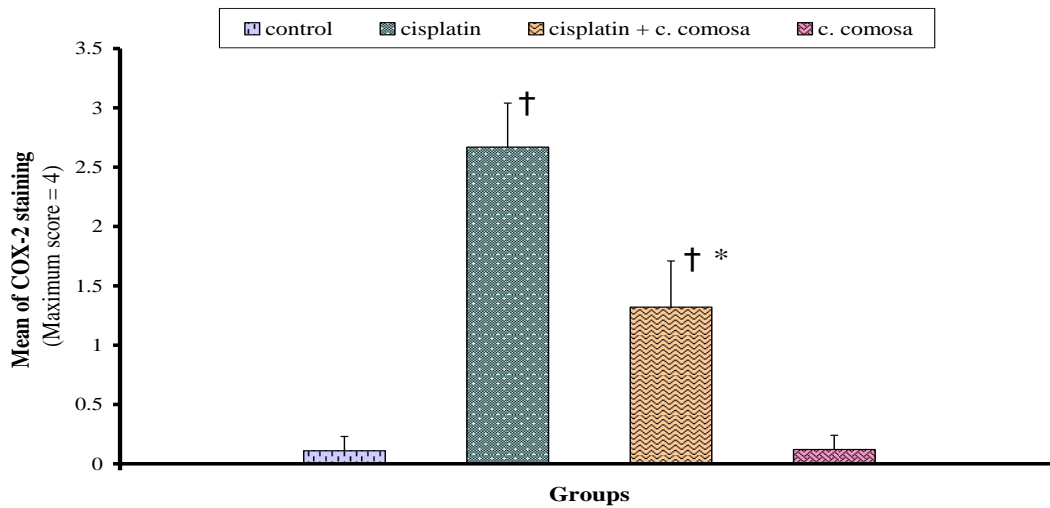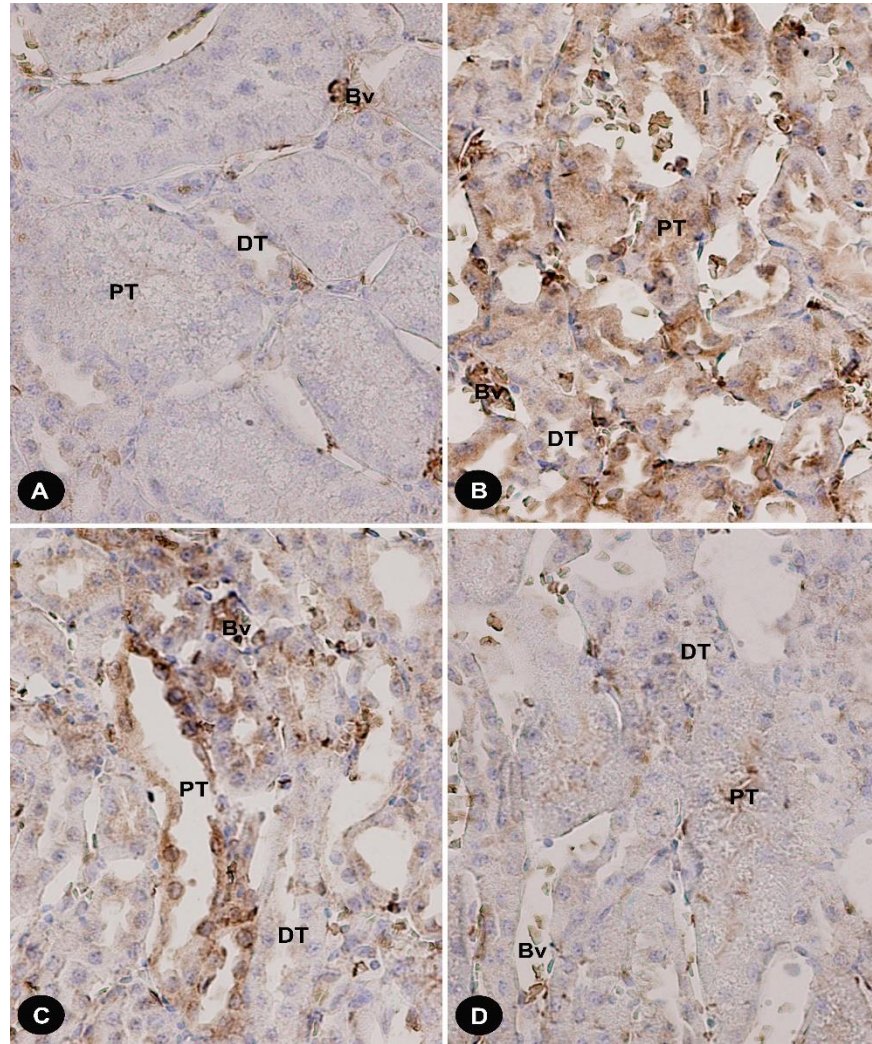Einhorn L, Donohue J (1977). Cis-diamminedichloroplatinum,

Figure 3. Higher magnification of the outer stripe of outer medulla of kidney, in cisplatin kidney (B), the intense of COX-2 expression were noted at proximal tubular cells (PT) as compared to control (A) and *C. comosa* groups (D). However, pretreatment with *C. comosa* (C) **s**howed markedly decreased stained at proximal tubular cells. The semi-quantitative evaluation of COX-2 immuno-staining (E), the extent of COX-2 expression was graded on area field 0.042 mm^2 in 4×20 fields per slide of outer stripe of outer medulla. Data represent mean ± SEM from all experimental groups ([†]P<0.05 vs. control; *P< 0.05 vs. cisplatin; DT, distal tubule; Bv, blood vessel; ×400).

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