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Original Article

The protective effect of hydroalcoholic *Citrus aurantifolia* peel extract against doxorubicin-induced nephrotoxicity

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Abstract

Background and purpose: Doxorubicin chemotherapy is a widely used treatment for various cancers, including breast, ovarian, and uterine cancers, among others. However, long-term use can cause nephrotoxicity side effects. Some citrus flavonoids have demonstrated nephroprotective activity; therefore, this study aimed to test the nephroprotective effectiveness of *Citrus aurantifolia* peel extract in protecting and reducing kidney damage caused by doxorubicin.

Experimental approach: *Citrus aurantifolia* peel was dried, ground, and extracted by ultrasonication (70% ethanol), then the extract was dried. Twenty-five female Sprague-Dawley rats were divided into 5 groups including the normal group (control), positive control (doxorubicin) group receiving doxorubicin at the repeated intraperitoneal (i.p.) dose of 4 mg/kg/day on days 2, 6, 10, and 14, and treatment groups receiving *Citrus aurantifolia* peel extract (CPE) with the doses of 100, 200, and 400 mg/kg/day orally for 14 days, and doxorubicin (4 mg/kg/day, i.p.) on days 2, 6, 10 and 14. On day 15, the rats were euthanized for the measurements of MDA, superoxide dismutase (SOD), catalase, kidney function (measuring blood urea nitrogen (BUN), creatinine, albumin serum levels), and renal histopathology.

Findings/Results: The CPE yield was 16.13%. CPE could significantly reduce the levels of MDA, and increase SOD and catalase activities compared with the doxorubicin-induced nephrotoxic model. CPE could increase renal function by reducing BUN and creatinine levels, increasing albumin, and improving the histopathology of the kidney.

Conclusion and implications: CPE has a potential effect as nephroprotective against doxorubicin-induced toxicity in renal through antioxidant capacities and increased renal function.

Keywords: Citrus aurantifolia peel; Doxorubicin; Nephrotoxicity; Oxidative stress.

INTRODUCTION

Cancer is a disease that arises due to the uncontrolled growth of abnormal cells in the body and can attack and move between cells and tissues (1). According to World Health Organization data, Indonesia was ranked the 8th number in the world in 2020 with the cases of death caused by cancer, a total of 234,511 cases. The most common type of cancer found in Indonesia is breast cancer (more than 60,000 cases) (2). With chemotherapy being one of the therapies that is often an option for treating cancer, other therapeutic methods consist of surgery, immunotherapy, hormone therapy, and irradiation, which are adjusted to the stage of cancer. Doxorubicin is one of the most widely used cancer drugs as chemotherapy (3).

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Doxorubicin is an anthracycline class that can be given alone or combined with other chemotherapeutic agents. Doxorubicin is most widely used in treating several types of cancer such as lymphomas and soft tissue sarcoma, breast, genitourinary, ovarian, gastrointestinal, liver, and hematologic cancers because it has a broad spectrum of action. However, the long-term use of doxorubicin causes several side effects, so the duration of usage should be limited. One of the most common side effects is nephrotoxicity which is characterized by glomerulosclerosis, interstitial fibrosis, decreased glomerular filtration rate, albuminuria, hypoalbuminemia, increased creatinine and urea in the blood, and changes in renal histopathology (4,5). Nephrotoxicity occurs when the detoxification and excretory roles of kidneys do not function properly because of the damaging effect of exogenous or endogenous toxins (6). As regards doxorubicin can bind to iron to form chelates resulting in the formation of free radicals, and subsequently cellular damage (7,8), the mechanism of doxorubicin causing toxicity is performed further through an enzymatic process by flavoenzymes with the help of NADPH to form the semiquinone free radicals (9). It has been reported that the prevalence of nephrotoxicity related to the administration of chemotherapy drugs in cancer patients in the Hospital Cancer Unit is 35.9% (10), which is comparable to research conducted in Indonesia, which recorded a prevalence of 38.4% (11). The long-term use of doxorubicin can cause nephrotic syndrome with renal lesions and focal segmental glomerular sclerosis (12). A case reported the occurrence of collapsing glomerulopathy associated with the use of doxorubicin in patients with acute myeloid leukemia and lymphoma (13). Another study reported a patient having the stages of kidney disease including I (42.7%), II (28.6%), and III (4.9%) among 206 adult cancer patients who received chemotherapy (10). The side effects can affect treatment, the quality of life, and patient survival (14). Therefore, by administering compounds with antioxidant activity derived from natural ingredients such as nephroprotectants (13,15) it is necessary to make efforts to reduce the doxorubicin-induced kidney damage.

Citrus sp. has an abundance of flavonols, namely hesperidin (58.66%), didymin (3.46%), hesperetin (1.87%), and rutin (36.67%), with its utilization being only for spices, although it is also used for medicinal herbs (16). There is the highest concentration of flavonoids in the skin of citrus, which has health benefits. Based on their antioxidant activity, the flavonoids can prevent the formation of free radicals and are protective against cardiovascular disease, inflammation, allergies, and platelet aggregation (17). In addition, some flavonoids such as hesperidin and naringenin owned by Citrus sp., especially in the skin have shown nephroprotective properties (18,19). *Citrus aurantifolia* as one of the citruses is widely available and consumed in Indonesia. Many benefits of *Citrus aurantifolia* make its processing high resulting in new problems in the processing of peel waste, which is not optimal. *Citrus aurantifolia* peel is likely to be a source of environmental issues due to microbial fermentation and decomposition processes (20). Thousands of tons of citrus peels produced are generally considered agro-industrial waste. Interestingly, citrus peels are valuable by-products that can be used in the pharmaceutical industry due to having good sources of natural flavonoids (21).

Based on the background explained above, it is important to utilize citrus waste to make it more beneficial. In other words, it is possible to exhibit more advantages of citrus peel through preclinical experiments in animal models. In addition, the nephroprotective effects of (CPE) on antioxidant capacity including malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) and kidney function including creatinine, blood urea nitrogen (BUN), and albumin levels, as well as kidney histopathology in doxorubicin-induced nephrotoxicity in rats were unclear. Therefore, the current study was carried out to examine the nephroprotection of hydroalcoholic CPE in protecting and reducing kidney damage against nephrotoxicity induced by doxorubicin administration in female rats.

MATERIALS AND METHODS

Drugs and chemicals

Doxorubicin was obtained from local pharmaceutical (Global Onkolab Farma), Ethanol, Tween 80, EDTA, and phosphate buffer (pH 7) were provided by Brataco Chemical (Indonesia). Tetraethoxypropane (standard for MDA), trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (USA). RANDOX (UK) and Elabscience (USA), respectively, provided assay kits for SOD (RanSOD) and CAT. Colorimetric urea, creatinine, and albumin assay kits were purchased from Biolabo SAS, France.

Preparation of CPE

Citrus aurantifolia fruits were collected from a plantation in Bogor, Indonesia, and identified by a botanist. The fruits were washed, peeled, and air-dried. After that, *Citrus aurantifolia* peel powder (100 g) was extracted by ultrasonication for 30 min at 40 °C with a frequency of 50 kHz using 70% ethanol. The extraction was carried out 3 times. Then, all extracts were evaporated to dry.

Experimental animals

Sprague-Dawley strain female white rats, 10 weeks of age and weighing 150-200 g, provided from commercial breeding rats and mice (Bogor, Indonesia) were used in this study. The rats were housed in the Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Pancasila University to acclimate for a week, with controlled conditions including a room temperature of 25 ± 3 °C, air ventilation for 11-13 times per day, humidity of 65 ± 10%, and 12-h illumination per day (06:00 am -06:00 pm). Standard pellets and water were free access for the animals. All animal experiments were approved and permitted by the Health Research Ethics Committee of the Faculty of Medicine, University of Indonesia (ethical No. 1043/UN2.F/ETIK/PPM/00.02/ 2021). In addition, efforts were made to minimize suffering and pain in animals.

Experimental design

To induce nephrotoxicity doxorubicin at a dose of 4 mg/kg, twice/week for 2 weeks was used which was previously documented to achieve nephrotoxicity and be harmful to the kidney (22,23). In the current study, 25 rats were divided into 5 groups (n = 5) as follows: group 1 (control group) without receiving any treatments; group 2 as a positive control (doxorubicin group) receiving doxorubicin at the repeated doses of 4 mg/kg intraperitoneally on days of 2, 6, 10 and 14; and the groups 3-5 receiving CPE at the doses of 100, 200, and 400 mg/kg/day orally for 14 days (24), and doxorubicin (4 mg/kg/day intraperitoneally on days 2, 6, 10 and 14) considered CPE 100 + doxorubicin, CPE 200 + doxorubicin, and CPE 400 + doxorubicin. Finally, on the 15th day, euthanasia was carried out, and all animals were dissected to take blood samples and remove kidneys. Plasma sample was used to measure MDA levels. Also, kidney tissues were applied to measure SOD and CAT activities and evaluate renal histopathology. The levels of creatinine, BUN, and albumin were measured in serum samples.

Serum, plasma, and tissue isolation

Blood samples were transferred to clean tubes containing K_3EDTA to measure MDA levels in the harvested plasma samples. Another part of blood was also considered to obtain serum, which was used for BUN, creatinine, and albumin measurement. In addition, both kidneys removed were weighed, and then the ratio of kidney weight to body weight was calculated. Kidney tissue was then cleaned with saline solution and then crushed using a mortar in phosphate buffer (pH 7.4, 0.01 M) in an ice bath (cold), with a ratio of 10 mL (phosphate buffer):1 g (kidney tissue). The homogenized kidney was centrifuged at 3000 rpm for 10 min, and the supernatant was transferred into a clean tube and used for SOD and CAT measurements.

Antioxidant capacity assay

MDA levels in plasma were measured according to the TBA assay (25). Briefly, the plasma (200 µL) was added to 20% TCA (1.0 mL) and 0.67% TBA (2 mL). The mixture was homogenized and heated for 10 min over a water bath. After cooling, the mixture was centrifuged for 10 min at 3000 rpm, and the supernatant was collected. Finally, a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) was used to detect the pink supernatant absorbance at 532 nm (26). The measurement

of SOD and CAT activities in the homogenized kidney was performed according to the manufacturer's protocol in the assay kits.

BUN, creatinine, and albumin assay

For the measurement of BUN, 5 µL of serum sample was added to 1000 µL of reagent R1a (995 µL of R1 and 5 µL of R2) and mixed. After 4 min, 1000 µL of R3 was added to the mixture and left for 8 min. Finally, the absorption was read at 600 nm.

To measure creatinine, 100 µL of serum sample was added to 1000 µL of reagent R1a (500 µL of R1 and 500 µL of R2) and mixed. The first (A1) and second (A2) absorptions were read after 30 s and 2 min at 490 nm against aquadest, respectively.

To measure albumin 5 µL of serum sample was added to 1000 µL of reagent R1a (500 µL of R1 and 500 µL of R2) and mixed. After changing the mixture color to green-blue, the absorbance was read at a wavelength of 630 nm.

Histopathological assay

Histology assay was prepared in several stages including fixation, dehydration, clearing, trimming, embedding, blocking, cutting/sectioning, staining with hematoxylin-eosin, and mounting. Qualitatively, the histopathological assay was evaluated for histopathological structural changes in the proximal convoluted tubule such as tubular cell necrosis, dilatation, vacuolization, cast formation, and loss of brush border. Semiquantitatively, 100 proximal tubules were investigated at 10× magnification in the outer border of the medulla and the inner cortex. The number of damaged tubules among 100 tubules was counted, and the kidney damage level based on the tubular damage percentage including 0 (0%), 1 (1%-10%), 2 (11%-25%), 3 (26%-50%), 4 (51%-75%), and 5 (> 75%) was determined.

Statistical analysis

For statistical analysis, SPSS version 25 was used. The data obtained were tested using the Shapiro-Wilk normality and the Levene homogeneity tests. Accordingly, the data were normally distributed, homogeneous, and parametric. All data were presented as mean ± SD. The statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by the least significant differences (LSD) test. *P*-values < 0.05 were considered statistically significant.

RESULTS

Phytochemical screening of CPE

The CPE yield was 16.13%. As shown in Table 1, phytochemical screening of CPE confirmed the presence of alkaloids, saponins, phenolic compounds, tannins, flavonoids, triterpenoids, and glycosides.

Effect of CPE on the MDA plasma level and the SOD and CAT enzyme activity in kidney

The plasma levels of MDA in all groups were exhibited in Fig. 1A. According to the results, there was an increase in the level of MDA in the doxorubicin group compared with the control group. Fig.1A revealed that the CPE at doses 100, 200, and 400 mg/Kg decreased MDA levels by 9.84%, 40.43%, and 74.5%, respectively. The 100 mg/Kg dose of CPE showed a significant difference as compared to the control group, but had no significant difference compared with the doxorubicin group, so it can be interpreted that the CPE at the dose of 100 mg/Kg was not able to reduce MDA levels. The dose of 200 mg/Kg had significant differences with the control and doxorubicin groups, so it can be stated that the dose of 200 mg/Kg was able to reduce MDA levels, but not at the levels of the control group. In this study, CPE at the dose of 400 mg/Kg had a significant difference with the doxorubicin group and had no significant difference compared to the control group, so CPE at the 400 mg/kg dose could reduce MDA levels closely to that of the control group (Fig. 1A).

Table 1. Phytochemical screening of *Citrus aurantifolia* peel extract.

Active compound	Result
Saponins	+
Alkaloids	+
Tannins	+
Phenolic compounds	+
Flavonoids	+
Triterpenoids/steroids	+
Glycosides	+

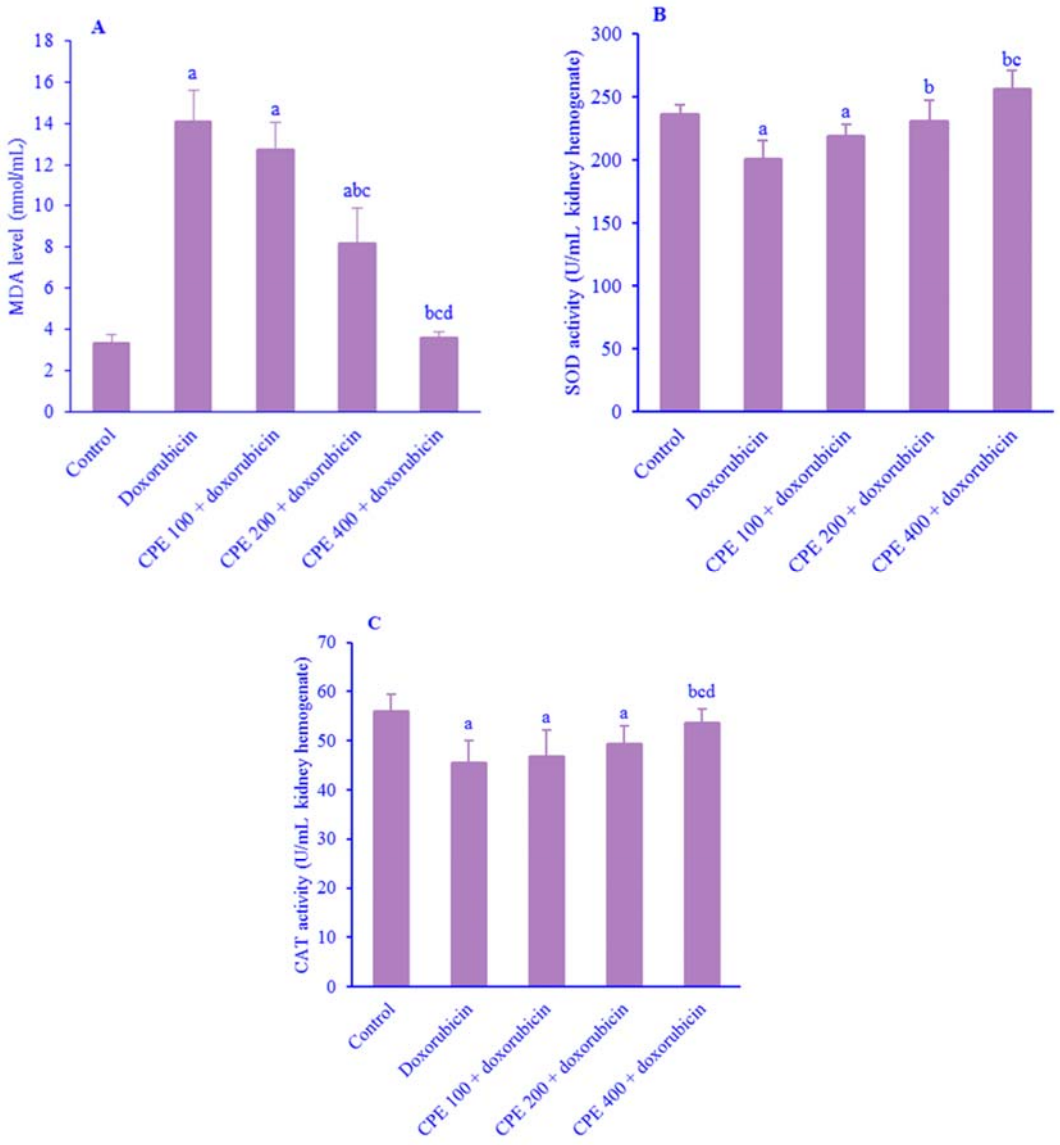


Fig. 1. The effect of CPE (100, 200, and 400 mg/kg) on the (A) MDA plasma level; (B) SOD activity; and (C) CAT activity in the kidney tissue in the groups receiving doxorubicin. The doxorubicin group as the positive control group received doxorubicin at the repeated doses of 4 mg/kg/day on days 2, 6, 10, and 14. Data were presented as mean ± SD. $^{a}P < 0.05$ demonstrates significant difference compared with the control group; $^{b}P < 0.05$ versus the doxorubicin group; $^{c}P < 0.05$ versus CPE 100 + doxorubicin group; $^{d}P < 0.05$ versus CPE 200 + doxorubicin group. CPE, *Citrus aurantifolia* peel extract; CAT, catalase; MDA, malondialdehyde; SOD, superoxide dismutase.

Furthermore, the results of measuring the activity of SOD enzyme in kidney tissue in the experimental groups were illustrated in Fig. 1B. The results showed that SOD activity in the doxorubicin group decreased compared to the control group, significantly. Treatment with CPE at the doses of 100 and 200 mg/Kg for 14 days increased SOD activity compared to the doxorubicin group, although it, especially the 100 mg/kg dose, did not reach the levels of SOD of the control group. CPE at the dose of 400 mg/Kg could significantly increase SOD activity compared to the doxorubicin group and CPE 100 + doxorubicin groups so that it was closer to the control group (Fig. 1B).

Figure 1C shows the levels of CAT enzyme activity among the experimental groups. The measurement of CAT activity revealed that

doxorubicin alone reduced CAT activity significantly compared to the control group, The 100 and 200 mg/Kg doses of CPE had significant differences compared with the control group and had no significant differences compared with the doxorubicin group, so it could be interpreted that the doses of 100 and 200 mg/Kg CPE were not able to increase CAT activity. The 400 mg/Kg dose of CPE had a significant increment compared with the doxorubicin group. On the other hand, no significant difference was observed in CAT activity in the CPE 400 + doxorubicin group compared to the control group, so the 400 mg/kg dose of CPE could increase CAT activity to approach the control group (Fig. 1C).

Effect of CPE on body weight and kidney weight/body weight

In this study, all animals were weighed before (day 0) and after the treatment (day 15) which are presented in Fig. 2A. The results showed that rats given repeated doses of 4 mg/kg doxorubicin on days 2, 6, 10, and 14 were reported to be inactive and had decreased appetite resulting in significant body weight loss. In the groups receiving CPE treatment significant increases in body weight at doses 200 and 400 mg/kg (Fig. 2A) were observed. This subject proved that CPE can prevent body weight loss in rats receiving the chemotherapy drug doxorubicin.

The ratio of kidney weight to body weight (KW/BW) was calculated based on the rat body weight on day 15 and presented in Fig. 2B. There was a significant increment in KW/BW in the doxorubicin group compared with the control group. In contrast, the groups receiving CPE exhibited a decrease in KW/BW compared to the doxorubicin group, which were significant at the doses of 200 and 400 mg/kg. CPE at the dose of 400 mg/kg could repair kidney damage indicated by a decrease in KW/BW at that dose compared to the other doses, significantly.

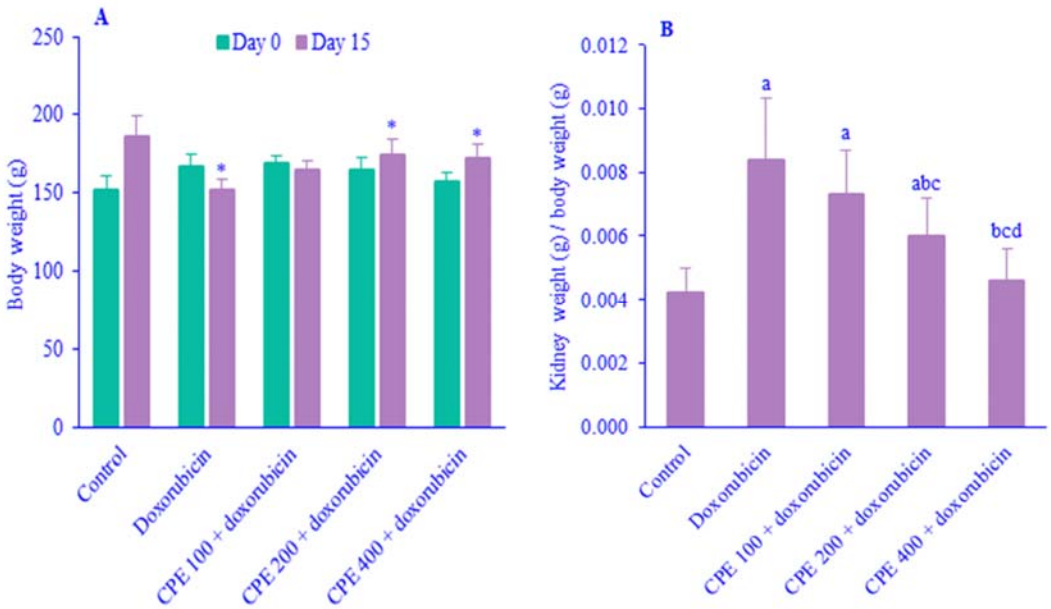


Fig. 2. The effect of CPE (100, 200, and 400 mg/kg) on the (A) body weight and (B) kidney weight/body weight in the groups receiving doxorubicin. Body weight was recorded on days 0 (before treatment) and 15 (after treatment). The doxorubicin group as the positive control group received doxorubicin at the repeated doses of 4 mg/kg/day on days 2, 6, 10, and 14. Data were presented as mean ± SD. $^{*}P < 0.05$ demonstrates significant difference compared with day 0; $^{a}P < 0.05$ versus control group; $^{b}P < 0.05$ versus doxorubicin group; $^{c}P < 0.05$ versus CPE 100 + doxorubicin group; $^{d}P < 0.05$ versus CPE 200 + doxorubicin group. CPE, *Citrus aurantifolia* peel extract.

The effect of treatment with CPE on the serum levels of BUN, creatinine, and albumin

Induction with doxorubicin resulted in a decrease in renal function indicated by the serum levels of BUN, creatinine, and albumin on day 15, as shown in Fig. 3. Based on the results obtained in the study, the serum levels of BUN and creatinine as the parameters of renal function showed very high values of 254.61 and 9.86 mg/dL in the doxorubicin group, respectively, which were significant compared with control group (Fig. 3A and 3B). CPE administration at doses 200 and 400 mg/kg showed improvement in kidney function, which could be observed as a gradual decrease in the serum levels of BUN and creatinine compared with the doxorubicin group, significantly. In addition, the 400 mg/kg dose of CPE showed a significant decrease in the serum levels of BUN and creatinine compared to the group receiving the 200 mg/kg dose of CPE (Fig. 3A and 3B).

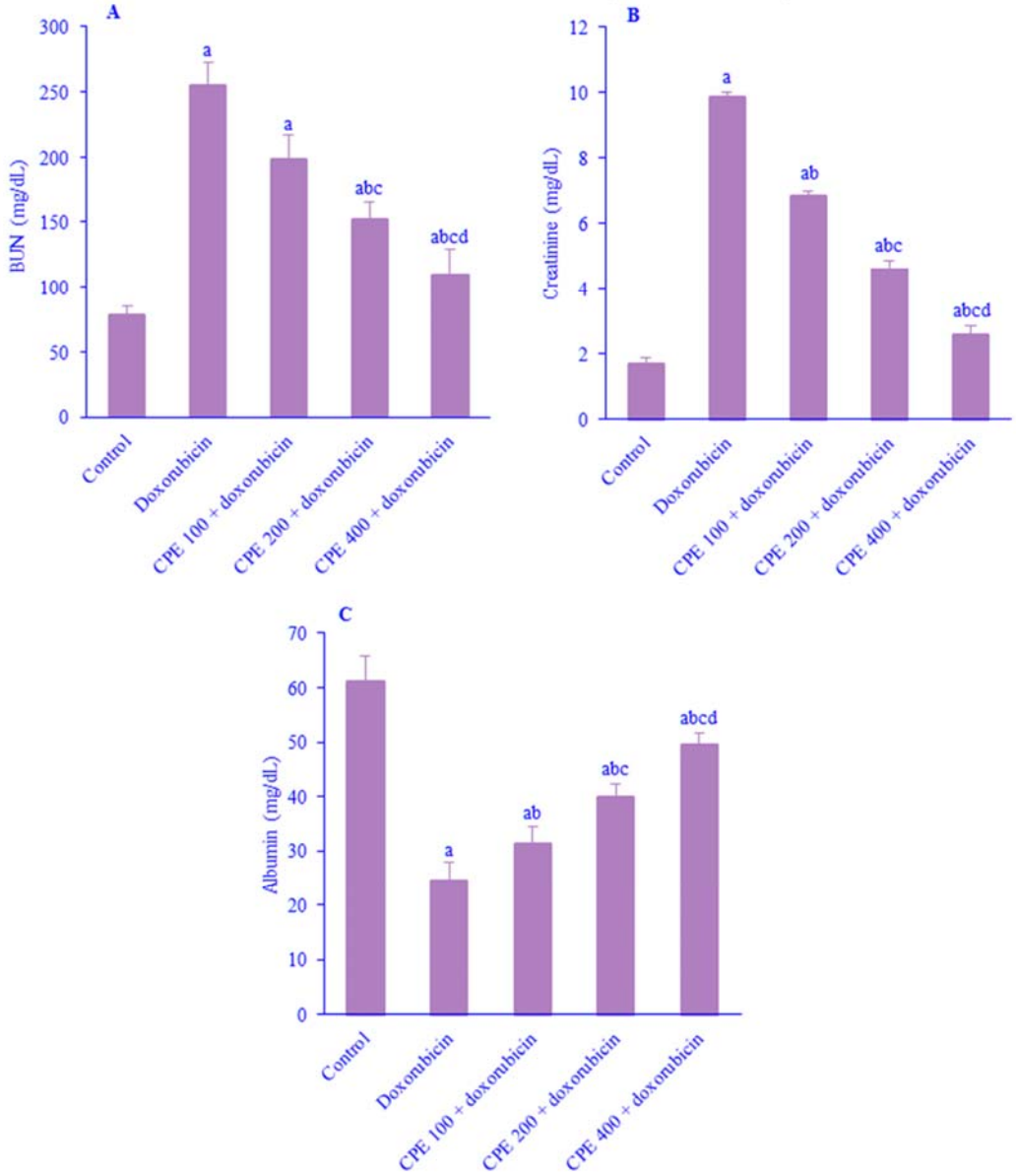
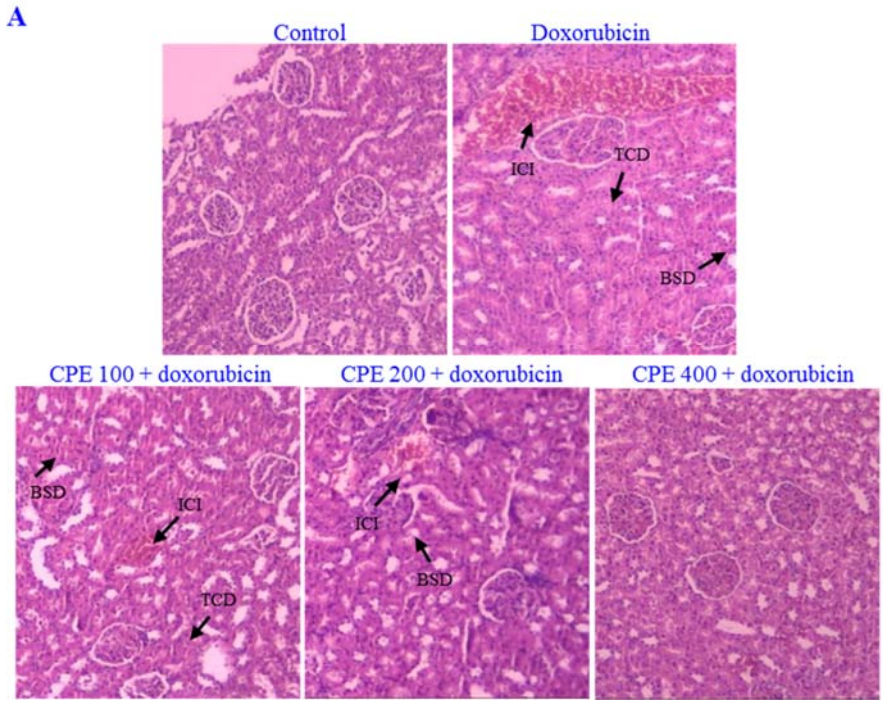


Fig. 3. The effect of CPE (100, 200, and 400 mg/kg) on the serum levels of (A) BUN; (B) creatinine; and (C) albumin in the groups receiving doxorubicin. The doxorubicin group as the positive control group received doxorubicin at the repeated doses of 4 mg/kg/day on days 2, 6, 10, and 14. Data were presented as mean ± SD. $^{a}P < 0.05$ demonstrates significant difference compared with the control group; $^{b}P < 0.05$ versus the doxorubicin group; $^{c}P < 0.05$ versus CPE 100 + doxorubicin group; $^{d}P < 0.05$ versus CPE 200 + doxorubicin group. CPE, *Citrus aurantifolia* peel extract; BUN, blood urea nitrogen.

The results of measured serum albumin levels in the study showed that rats given doxorubicin alone had a significant decrease in the serum albumin levels in comparison to the control group, indicating a reduction in kidney function (Fig. 3C). In the groups receiving CPE treatment, there was a significant increase in the serum albumin levels, indicating an improvement in kidney function compared with the doxorubicin group. The CPE 400 + doxorubicin group showed a more pronounced increase in the serum level of albumin than the CPE 100 + doxorubicin and CPE 200 + doxorubicin groups (Fig. 3C).

The effect of treatment with CPE on renal histopathology

The examination of rat kidney pathology began with an evaluation of kidney morphology and proceeded with a kidney histopathological investigation. The microscopic observations of renal tissues showed that rats in the control group showed normal structures and cells as well as found no abnormalities. The kidney view consists of the glomerulus surrounded by Bowman's capsule, the distal tubule, and the proximal tubule. The doxorubicin group indicated more severe damage than those treated with CPE (Fig. 4). The renal tissues of the doxorubicin group showed extensive necrosis, vascular congestion, inflammatory cell infiltration, tubular cell degeneration, and Bowman's space dilatation. There were minor areas of necrosis in the CPE-treated groups compared to the doxorubicin group without treatment. In the doxorubicin-induced rats given CPE at the dose of 100 mg/kg, blood vessel congestion, inflammatory cell infiltration, Bowman's space dilatation, and tubular cell degeneration were still visible. The group given doxorubicin and CPE at the dose of 200 mg/kg showed inflammatory cell infiltration, blood vessel congestion, and little tubular cell degeneration. Meanwhile, the group receiving the 400 mg/kg dose of CPE showed improvement in kidney function and almost normal kidney tissue structure with no blood vessel congestion and tubular cell degeneration. Also, less inflammatory cell infiltration was observed (Fig. 4).



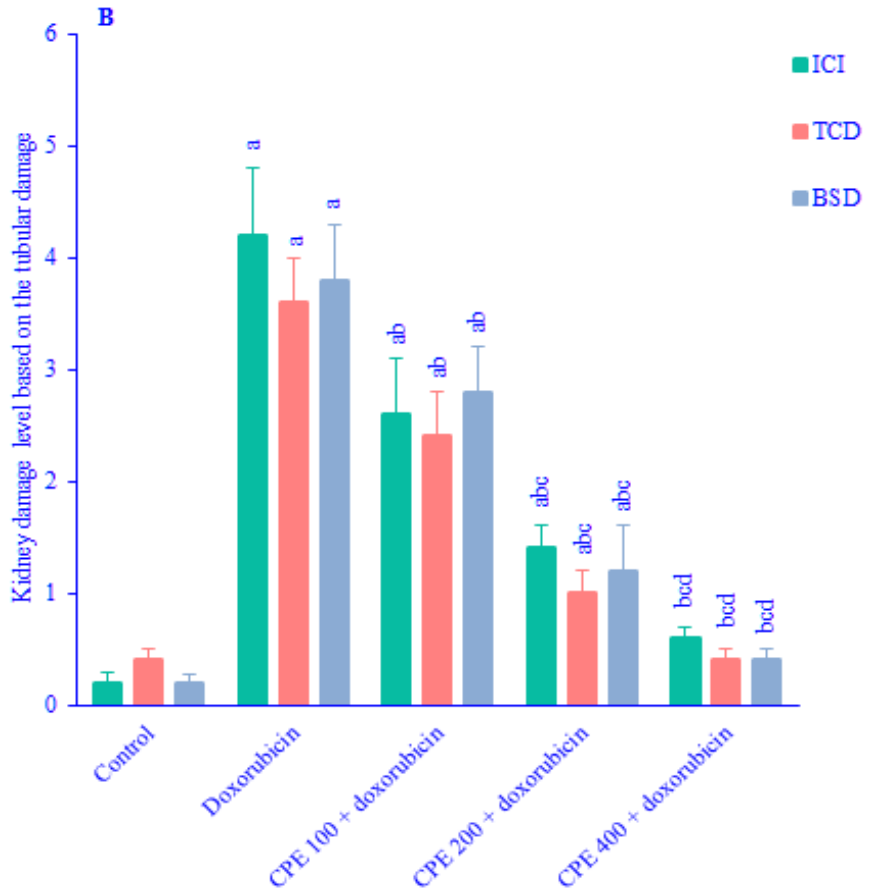


Fig 4. The effect of CPE (100, 200, and 400 mg/kg) on kidney damage in the groups receiving doxorubicin. (A) The histopathological images of kidney stained by hematoxylin & eosin (10× magnification) in all experimental groups and (B) the kidney damage level based on the tubular damage. The doxorubicin group as the positive control group received doxorubicin at the repeated doses of 4 mg/kg/day on days 2, 6, 10, and 14. Data were presented as mean ± SD. $^{a}P < 0.05$ demonstrates a significant difference compared with the similar factor in the control group; $^{b}P < 0.05$ versus the analogous factor in the doxorubicin group; $^{c}P < 0.05$ versus the analogous factor in the CPE 100 + doxorubicin group; $^{d}P < 0.05$ versus the analogous factor in the CPE 200 + doxorubicin group. CPE, *Citrus aurantifolia* peel extract; ICI, inflammatory cell infiltration; TCD, tubular cell degeneration; BSD, Bowman's space dilatation.

DISCUSSION

Doxorubicin-induced nephrotoxicity is considered a well-established and highly reproducible experimental model of kidney diseases (27). The damaging effect of doxorubicin-induced nephrotoxicity is primarily organized by the selective damaging of proximal tubule cells through mechanisms that continue to be the focus (28). The present study was offered to examine the CPE perspective to avert chemotherapeutics drug-induced renal toxicity, which might enhance the antitumor efficacy of doxorubicin.

Although doxorubicin is known to successfully kill cancer cells when used individually or in combination with another method, there are toxic effects on several organs such as the kidneys, heart, and liver (29,30). Therefore, the usage of doxorubicin is known to have limitations due to several acute and chronic side effects. Doxorubicin-induced kidney toxicity, acute kidney injury is characterized by damaged podocyte cells, so that their filtering function is disrupted, and metabolic waste such as urea and creatinine are accumulated in the body. Also, nephrons are damaged, resulting in proteinuria. Proteinuria can damage the tubules, characterized by increased urinary neutrophil gelatinase-associated lipocalin (NGAL) excretion, and cause interstitial fibrosis (31,32). Generally,

indicators used as markers for acute kidney injury are plasma urea and creatinine (33). Urine analyses indicate the acid-base balance and kidney functional capacity level, with increased albumin, urine creatinine, and urea being common indicators of treatment-induced kidney injury (34). The injection of doxorubicin causes a decrease in glomerular filtration rate in rats which is associated with increased serum creatinine, BUN, and urea (35). Current results are in agreement with previous results in other studies, which revealed that increased serum levels of biomarkers of renal damage are concurrent to impaired renal architecture and tubular blockade (36,37). In the current research, doxorubicin-induced nephrotoxicity was indicated by a considerable increase in BUN and creatinine serum levels, which was authenticated by histopathological changes compared to the control group.

This study showed that the 100 mg/kg dose of CPE had a weak nephroprotective effect but the amelioration of doxorubicin-induced kidney damage was more prominent in rats treated with the 400 mg/kg dose of CPE. The present research demonstrated that CPE at the highest dose (400 mg/kg) reduces the serum levels of BUN and creatinine to near-normal values, although they have not reached normal values. The decrease in BUN and creatinine levels due to the administration of CPE indicated the success of CPE in preventing further kidney damage. In line with the current findings, Wang *et al*. reported that the 200 and 400 mg/kg doses of *C. aurantium* markedly normalized plasma and urinary sodium, potassium, and creatinine levels, and the amelioration of cisplatin-induced kidney damage was more prominent in rats treated with 400 mg/kg of *C. aurantium* (24).

While doxorubicin decreases the levels of major antioxidant enzymes, such as SOD and CAT (38), The most plausible mechanism of doxorubicin-induced renal damage engages the excessive production of oxidative stress and free oxygen radicals (39). Suggesting that agents with antioxidant capabilities may have potential protective roles against doxorubicin-induced toxicity, the oxidative stress response is a critical cause of doxorubicin-induced renal injury (40). Accordingly, our research showed that doxorubicin significantly decreased CAT and SOD activity levels, and increased MDA, which enhanced reactive oxygen species (ROS) generation and induced oxidative damage. The increased MDA levels demonstrated that doxorubicin accelerated the lipid peroxidation process. The oxidation of polyunsaturated fatty acids is the first step in the cell-damaging process known as lipid peroxidation followed by autocatalytic chain reactions. By neutralizing enzymes and receptors associated with the cellular membranes, MDA may also harm the membrane proteins by the polymerization of the membrane's constituent parts. The increase in MDA levels caused by doxorubicin had a toxic effect on the kidneys. Doxorubicin is able to form oxidation and reduction reactions, reversibly. Doxorubicin can bind to tubular cell membrane receptors and form semiquinone free radicals through an enzymatic process by flavoenzymes with the help of NADPH. In addition, doxorubicin is also able to bind to iron to form chelates, resulting in the formation of free radicals, and subsequently decreasing the antioxidant compounds with the occurrence of lipid peroxidation (41). ROS attacks the polyunsaturated fatty acids in membrane lipids, proteins and genetic material. SOD catalyzes the dismutation of the superoxide anion to hydrogen peroxide (H_2O_2), which is then detoxified to be H_2O by CAT (42). The increased lipid peroxidation is evidence supporting the involvement of oxidative stress in tissues, and the activation of free radical precursors in all tissues (43). Antioxidants including both enzymatic (SOD, glutathione peroxidase, and CAT) and non-enzymatic (flavonoids) agents can provide the necessary defense system against oxidative stress without compromising the clinical efficacy of doxorubicin (44).

The present study exhibited CPE can inhibit lipid peroxidation. The administration of CPE at the doses of 100 and 200 mg/Kg reduced MDA levels, while the dose of 400 mg/Kg decreased MDA levels closely to the control group. In this study, the antioxidant capacity of CPE was also assessed by evaluating the activity of the SOD and CAT enzymes. The high doses of CPE increased the activity of the

SOD and CAT enzymes. In this research, MDA levels and SOD activity in groups receiving CPE at the doses of 200 and 400 mg/kg showed significant differences compared with the group receiving doxorubicin alone. The best CAT activity was exhibited at the dose of 400 mg/kg, indicating that the antioxidant ability of CPE is high at the doses of 200 and 400 mg/kg. In confirming the current research results, the previous study reported that pretreatment with *C. aurantium* extract at the dose of 300 mg/kg dose reduced MDA levels by 31% and at the dose of 100 mg/kg significantly decreased the MDA cardiac levels by 22% in $K_2Cr_2O_7$-induced toxicity (45). The decrease in MDA levels and the increase in the activity of SOD and CAT enzymes in the groups receiving *C. aurantium* extract could occur due to the decreased free radicals resulting from the presence of phenolic content such as flavonoids which act as antioxidants (46). Flavonoids can inhibit enzymes such as xanthine oxidase and protein kinase C, which are responsible for producing superoxide anion radicals. In addition, flavonoids also inhibit cyclooxygenase, lipooxygenase, monooxygenase, microsomes, glutathione S-transferase, and NADH oxidase enzymes, all of which are involved in the formation of ROS. When free radicals can be suppressed, oxidative stress does not occur so that plasma MDA levels do not increase, and SOD and CAT enzyme activities do not decrease (21,47). Notably, CPE has an antioxidative role and scavenges doxorubicin-induced oxygen radicals implying one of the main mechanisms in which CPE could protect renal tubular cells against doxorubicin nephrotoxicity.

In this study, the changes in body weight of rats were observed. Body weight data were used to evaluate the pathological state caused by the harmful effects of doxorubicin. Decreased body weight and increased mortality of animals were toxic effects associated with the administration of doxorubicin. Giving doxorubicin 3 mg/kg/week can cause body weight loss due to decreasing appetite (48). Also, rats exposed to doxorubicin had a decrease in the ability to concentrate urine and papillary hypertonicity known as the main cause of body weight loss in rats (31). In this research, the administration of doxorubicin 4 mg/kg repeated doses on days 2, 6, 10, and 14 caused mice to be inactive and experience a decrease in appetite resulting in a severe reduction in body weight, while CPE at the doses of 200 and 400 mg/kg rescued the observed body weight loss. Ramalingayya *et al*. found that doxorubicin alone comparatively lowered the average body weight, but the treatment with rutin as a protective agent could reverse doxorubicin-induced body weight loss (49). Literature has documented that rutin was identified as one of the active components of CPE (16). Therefore, it seems that rutin as effective compound could overcome body weight loss in doxorubicin chemotherapy.

The assessment of kidney function improvement was carried out by giving CPE. While, in the presence of kidney toxicity, kidney weight increased in proportion to the toxicity occurring in the kidney. KW/BW ratio is one of the parameters that can be used to determine the presence of kidney toxicity. In mice exposed to chemotherapy drugs such as doxorubicin, there was a decrease in the ability to concentrate urine and papillary hypertonicity which is known to be the main cause of body weight loss in mice. Therefore, the increase in kidney weight is not due to body weight gain, but because of the enlargement of the tubular cells in the kidney exposed to doxorubicin. The increase in kidney weight correlated with an increase in serum creatinine and BUN values (50).

This research showed that doxorubicin increased the serum level of BUN not only due to a decrease in kidney function but also due to reabsorption occurring in the Henle loop. Under normal circumstances, urea is only reabsorbed in the distal and proximal tubules, whereas as much as 40%-50% of the filtered urea is reabsorbed in the proximal tubule (51). A study conducted by Refaie *et al*. revealed that a 15 mg/kg single dose of doxorubicin resulted in a significant increase in BUN levels as much as 251.8 mg/dL (52). The current study showed that BUN levels decreased after being given CPE, which pointed to the nephroprotective effect of CPE against doxorubicin-induced nephrotoxicity. Therefore, it can be said that CPE protects the kidneys from various kidney disorders caused by toxins such as doxorubicin.

Creatinine is considered a specific marker of kidney function, even so, a significant increase in creatinine levels can only be seen when the value of the glomerular filtration rate is reduced by about 50%. Several factors can cause an increase in creatinine levels in the blood, including dehydration, excessive fatigue, and the use of drugs that are toxic to the kidneys (53).

Albumin can be considered a parameter in measuring nephrotoxicity induced by chemotherapy drugs. The drugs can lead to necrosis of podocyte cells involved in albumin filtration. The presence of necrosis in the podocyte cells causes larger gaps in the glomerulus so that proteins with a larger molecular weight such as albumin can pass through the glomerular filtration, as a result, the values of serum albumin decrease (54).

According to the histopathological findings, the kidneys in the control group had normal histological structures, while the group receiving doxorubicin exhibited quite severe lymphocytic inflammatory cell infiltration, accompanied by vascular congestion and tubular cell degeneration. The histopathological changes in the kidney mainly occur in the proximal tubule because doxorubicin accumulates mostly in the proximal tubule (28,51). In the group given CPE at the dose of 400 mg/kg, the histopathological structure gradually resembled the control group by showing an improvement in the size of the Bowman's space and the absence of inflammatory cell infiltration, which indicated that CPE reduced renal histological changes caused by doxorubicin.

The morphological effects of doxorubicin-induced renal damage were reported by Bilgic *et al.* who observed tubular degeneration, medullary congestion/bleeding, tubular dilation and vacuolization, and cell infiltration (29). El-Sheikh *et al*. also reported the presence of Bowman's space dilatation, tubular degeneration and dilatation, and the presence of protein casts after doxorubicin administration (36). All metabolic processes in the body end with the excretion process in the kidneys. The products of metabolism undergo filtration in the glomerulus and reabsorption in the proximal tubule, Henle loop, and distal tubule, which continue to the collecting tubule to be excreted as urine. The proximal tubule is the most numerous part of the kidney and the most susceptible to damage in nephrotoxic cases. The kidneys consist of long-chain unsaturated fatty acids, making them vulnerable to damage caused by toxic substances (55). Doxorubicin induces renal injury by semiquinone reactive intermediates formed during oxidative stress (56). The anthracycline group was reported to form semiquinone radical intermediates which react with molecular oxygen to form ROS, and then interact with cell macromolecules resulting in cytological damage (57). Antioxidant compounds such as flavonoids at high concentrations counteracted free radicals (58). The results demonstrated that all doses of CPE gradually improved kidney histopathological changes induced by doxorubicin. Interestingly, the 400 mg/kg CPE exhibited renal histological improvement close to normal against doxorubicin toxicity.

Our findings showed that CPE mitigated renal impairment caused by doxorubicin by reducing oxidative stress. In addition, CPE shielded renal cells against doxorubicin-induced damage. The characteristics can propose CPE as a prospective option for protecting against chemotherapy-induced renal toxicity in cancer patients and a promising protective medication against renal toxicity. Additionally, our data supported the critical role of oxidative stress in doxorubicin-induced nephrotoxicity, paving the path for the development of effective medicaments to combat the side effects of chemotherapy drugs.

CONCLUSION

In conclusion, this study showed that the CPE has a nephroprotective effect against doxorubicin by increasing antioxidant capacity in kidney tissue including reducing MDA levels, increasing the activity of SOD and CAT enzymes, and increasing kidney function by lowering the serum levels of creatinine and BUN, increasing albumin serum level, and improving the renal histology. Further investigations are underway in our research team to address the pharmaceutical preparations with good bioavailability, effective and safe usage.

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Conflict of interest statements

All authors declared no conflicts of interest in this study.

Authors' contributions

N.M.D. Sandhiutami contributed to conceptualization design, supervised the experiments *in vivo*, data analysis, and participated in writing the report, writing and editing; Y. Desmiaty led the research, conducted the sample preparation and CPE extraction, screened the writing-review and editing, and contributed as corresponding author; P.D.U. Pitaloka and S. Salsabila conducted the experiments, the serum and kidney assessments, and data analysis. All authors approved the final version of the manuscript.

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