

Potential Roles of *Kleinhovia hospita* L. Leaf Extract in Reducing Doxorubicin Acute Hepatic, Cardiac and Renal Toxicities in Rats

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ABSTRACT

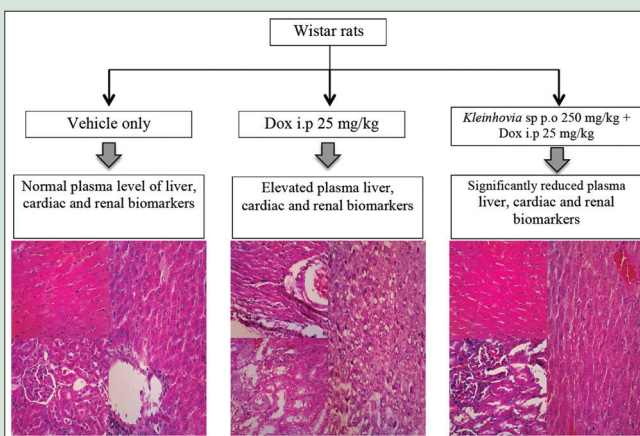
Background: Doxorubicin (DOX) is a potent chemotherapy agent; however, its use may lead to cardiac, hepatic, and renal dysfunction. *Kleinhovia hospita* L extract contains antioxidant compounds that have been shown to reduce chemical-induced hepatotoxicity. **Objectives:** This study aimed to examine the protective effects of *Kleinhovia* sp. extract to reduce DOX acute toxicities. **Materials and Methods:** Thirty male rats were assigned to the following groups: Group I as controls, Group II was given DOX i.p. injection (25 mg/kg); Groups III, IV, and V were treated with *Kleinhovia* sp. extract 100, 250, and 500 mg/kg orally for 5 days, respectively, prior to DOX i.p. injection. After 24 h, blood and organs were analyzed for biomarker levels and histopathological changes. **Results:** DOX treatment in Group II significantly increased creatine kinase-MB (CK-MB), aspartate transaminase (AST), alanine transaminase (ALT), and urea levels compared to controls. *Kleinhovia* sp. extract at any given dose significantly improved ALT and AST; yet, CK-MB levels only reduced with 250 mg/kg dose (Group IV). Urea and creatinine levels in *Kleinhovia* sp. groups were also lower compared to DOX-treated rats, but it was not significant. Histopathological analysis showed improved liver, heart, and renal tissue structures in *Kleinhovia* sp.-treated rats, especially at higher doses. **Conclusion:** *Kleinhovia* sp. extract at any dose given protected the rats from liver toxicity, but only at dose 250 mg/kg reduced cardiac toxicity. Although renal biomarkers were insignificantly lower, renal architecture was improved with *Kleinhovia* sp. treatment.

Key words: Acute toxicity, doxorubicin, *Kleinhovia* sp. extract

SUMMARY

- Doxorubicin (25 mg/kg) i.p injection led to elevated ALT, AST, CK-MB and urea levels in rats.
- At the given dose, doxorubicin induced pathological changes in cardiac, liver and renal tissues.
- Pretreatment with *Kleinhovia* sp. extract prior to doxorubicin injection significantly reduced the elevation of ALT, AST and CK-MB, especially at the dose of 250 mg/kg.
- Improvement in histological structures of cardiac, liver and renal tissues was

shown in *Kleinhovia* sp. (250 mg/kg) treated rats, indicating a protective effect of the extract on doxorubicin acute toxicity.



Abbreviations Used: DOX: Doxorubicin, CK-MB: creatine kinase-MB, AST: Aspartate transaminase, ALT: Alanine transaminase.

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INTRODUCTION

Doxorubicin (DOX) is a potent chemotherapy and widely used for many cancer types. However, it may lead to cardiac toxicity when used chronically or in a high dose, leading to myocardial dysfunction in cancer patients.^[1] Recently, DOX is also found to elicit hepatic and renal toxicity,^[2,3] raising more concern for DOX chemotherapy regardless its potential effects against cancer cells.

Kleinhovia hospita L is known as Paliase or Tahongai in Indonesia, and its leaves have been empirically used to treat hepatitis.^[4] *Kleinhovia* sp. hepatoprotection is mostly derived from its natural compounds, cycloartane triterpenoid alkaloids (Kleinhospitines A-D), which are shown to protect liver cells from H₂O₂ oxidative stress.^[5,6] Moreover, *Kleinhovia* sp. leaves also contain kaempferol 3-O-β-glucoside and eleuterol, which act as antioxidants.^[7] Indeed, hepatoprotective effects of *Kleinhovia* sp. have been demonstrated against paracetamol-induced

hepatotoxicity.^[8,9] It is hypothesized that antioxidant compounds in *Kleinhovia* sp. leaves may also protect cells from DOX toxicity, especially in the liver and possibly in the heart and kidney. Therefore, the aim of this study is to examine the effect of *Kleinhovia* sp. extract administration prior to i.p. injection of DOX on liver, cardiac, and renal acute toxicities.

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MATERIALS AND METHODS

Materials and extracts

DOX for injection (50 mg/25 ml) was purchased from Kalbe Farma (Makassar, Indonesia). Fresh *Kleinhovia* sp. leaves were obtained from Makassar, Indonesia, and dried in shade before cut into small pieces. Dried leaves were extracted with maceration in 70% ethanol for 3 days. Extract was thickened using a rotary evaporator and then prepared in three different concentrations (1%, 2.5%, and 5%) suspended in natrium carboxy-methyl-cellulose (NaCMC) 1%. Extract suspension was administered accordingly to obtain the doses of 100, 250, and 500 mg/kg body weight in rats. The dose of *Kleinhovia* sp. extract used in this study was selected based on a previous study showing hepatoprotective effects of *Kleinhovia* sp.^[10]

Animals

Male Wistar rats 200–250 g ($n = 30$) were caged with food and water *ad libitum*. Animals were cared for in accordance with the institutional standard for using experimental animals. Animals were adapted in the cage for 2 weeks before conducting experiment. The project was registered with ethical number of UH16101030.

Experimental design

Experimental protocol is depicted in Figure 1. Rats were assigned into one of the five groups. Blood samples were withdrawn 1 day before any treatment given to obtain baseline data. Group I was assigned as control rats and only given NaCMC 1% suspension for 5 days and then injected with NaCl 0.9% on the 5th day. Group II was given NaCMC 1% suspension for 5 days and a single shot of DOX (i.p. 25 mg/kg) on the 5th day. Groups III, IV, and V were, respectively, given *Kleinhovia* sp. extract orally with the dose of 100 mg/kg, 250 mg/kg, and 500 mg/kg for 5 days prior to DOX i.p. injection (day 5). After 24 h from DOX injection, rats were anesthetized with diethyl ether, and 3 ml of blood samples was obtained from rats' lateral tail veins using BD-Vacutainer® tube with ethylenediaminetetraacetic acid. Blood samples were centrifuged (Hettich®) at a speed of 2000 rpm for 25 min. The plasma was removed immediately and stored in -20°C until biomarker analysis was performed.

Biomarker analysis

Plasma biomarker creatine kinase-MB (CK-MB), aspartate transaminase (AST), alanine transaminase (ALT), urea, and creatinine were measured according to the diagnostic kit instructions specific for Humalyzer 3500 (Human®) instrumentation.

Histopathological examination

After withdrawing the blood, rats were euthanized with cervical dislocation. Heart, liver, and kidneys were carefully removed, fixated in 10% buffered formaldehyde, and embedded in paraffin. Sections of 5 μm thick were serially sliced with a microtome and stained with hematoxylin and eosin. Two subsequent sections of each organ sample were examined by an investigator blinded to the treatment groups using a microscope and photographed with a digital sight camera (Nikon Eclipse 50i).

Statistical analysis

Results are reported as mean $\pm$ standard error of mean for each group. Normal distribution was analyzed with 1-K sample Kolmogorov–Smirnov test. Normally distributed data were analyzed with one-way ANOVA followed by Tukey's honest significant difference *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline

All biomarker levels measured before treatments showed a normal range for rat plasma biomarkers and shown in Table 1.

Table 1: Baseline biomarker levels

Biomarkers	Range	Mean $\pm$ SEM
ALT	43.9–83.2	64.24 $\pm$ 6.01
AST	53.8–140.1	111.0 $\pm$ 14.44
CK-MB	55.0–179.0	103.22 $\pm$ 12.26
Urea	18.65–60.00	45.54 $\pm$ 4.51
Creatinine	0.23–1.14	0.74 $\pm$ 0.15

Data were obtained prior to initiation of any treatment. ALT: Alanine Transaminase; AST: Aspartate transaminase; CKMB: Creatine kinase-MB; SEM: Standard error of mean

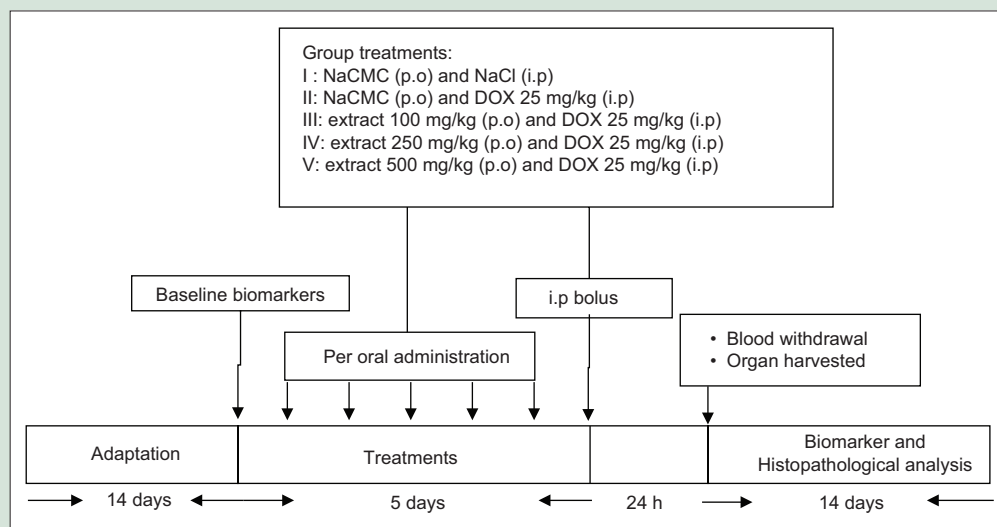


Figure 1: Diagram of experimental protocols. Animals were adapted for 14 days prior to *Kleinhovia* sp. extract administration for 5 consecutive days. At day 5, rats were injected with either saline or doxorubicin. After 24 h, blood was withdrawn and organs were harvested for further analysis

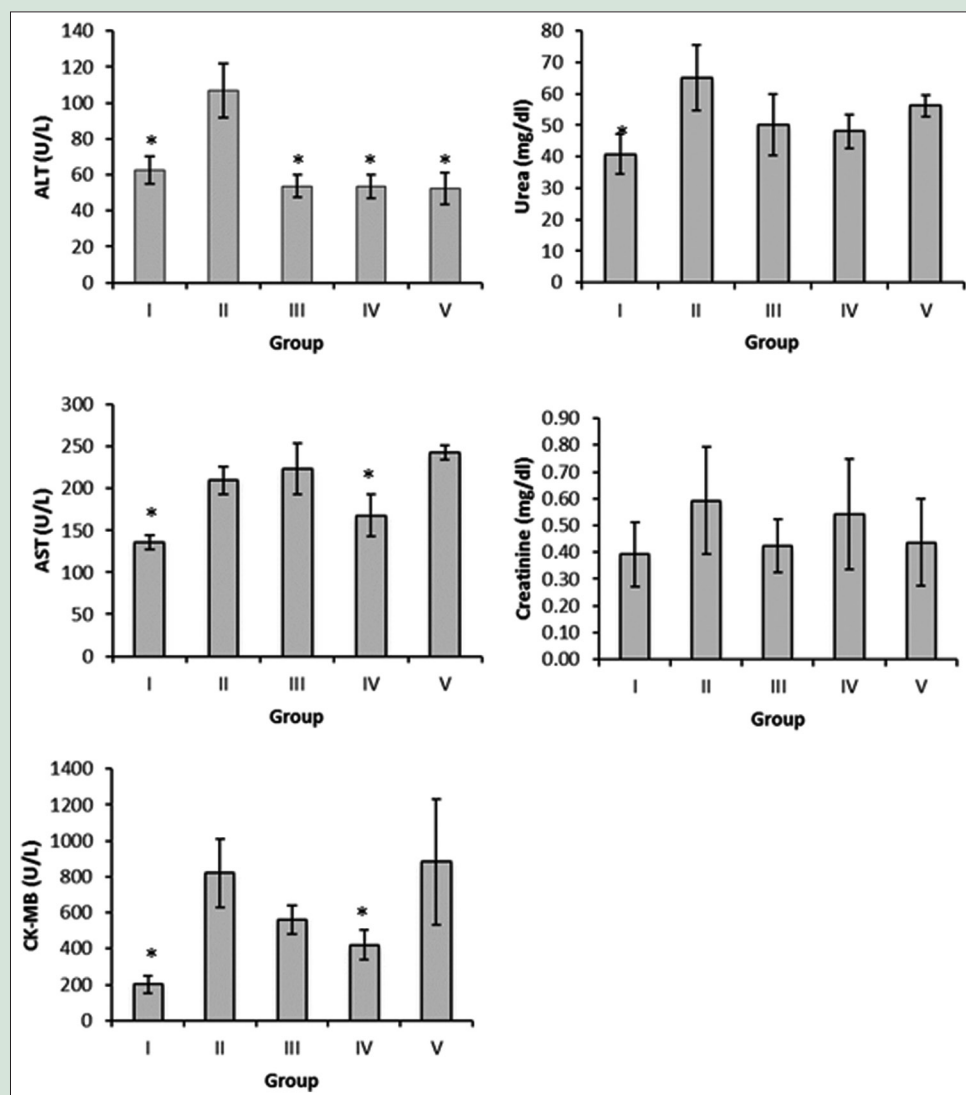


Figure 2: Levels of alanine transaminase, aspartate transaminase, creatine kinase-MB, urea, and creatinine in rats treated with doxorubicin only and *Kleinhovia* sp. Ethanolic extract. Data expressed in mean $\pm$ standard error of mean. Group I: controls, Group II: doxorubicin only, Group III: *Kleinhovia* sp. 100 mg/kg + doxorubicin; Group IV: *Kleinhovia* sp. 250 mg/kg + doxorubicin; Group V: *Kleinhovia* sp. 500 mg/kg + doxorubicin. * $P < 0.05$ compared with doxorubicin group (group II)

Biomarker analysis

Two parameters were used to indicate liver toxicity: ALT and AST. ALT is more sensitive and specific as an indicator of liver cell integrity and function, thus increased level of ALT is an excellent predictor to indicate liver damage. Meanwhile, AST is found equally abundant in the liver and cardiac cells, hence AST elevation may detect either liver or cardiac damage or both. The result shows that ALT levels in Group II significantly elevated 24 h after treated with DOX only. In contrast, rats treated with *Kleinhovia* sp. extract 5 days before i.p. injection of DOX did not experience ALT elevation, and this was shown in all *Kleinhovia* sp. groups independent of the dose given [Figure 2].

DOX-treated rats also had increased level of AST, twice as much as the normal controls ($P < 0.05$). Interestingly, AST elevation was also evident in groups that received *Kleinhovia* sp. extract at doses 100 and 500 mg/kg (Group III and V) in spite of a significant reduction in ALT levels in those groups. It is believed that the increased AST level was dominantly released from damaged cardiac cells rather than liver cells

as the ALT levels in those groups were near normal. In contrast, the rats treated with 250 mg/kg of *Kleinhovia* sp. extract had significantly lower AST level compared to DOX-treated rats [Figure 2]. This result indicates that *Kleinhovia* sp. extract was protective in hepatic cells from the dose of 100 to 500 mg/kg, but it was only protective in cardiac cells with the dose of 250 mg/kg.

This hypothesis is also supported by the result of CK-MB analysis, which is a sensitive and specific biomarker for myocardial infarction. It is shown that CK-MB level increased almost 4 folds after DOX injection [Figure 2]. With the doses of 100 and 250 mg/kg, *Kleinhovia* sp. extract was effective to reduce CK-MB level, but apparently, dose 250 mg/kg provided more protection. In contrast, the high dose of *Kleinhovia* sp. extract (500 mg/kg) failed to reduce cardiac biomarkers, both AST and CK-MB, and even led to an elevation of CK-MB marker in two out of six animals in the group.

For renal toxicity evaluation, two biomarkers were measured: urea and creatinine. Creatinine is a more specific marker to depict renal function compared to urea, but both markers are widely used in clinical setting

to monitor renal function. The data show that DOX injection led to significantly increased urea level but did not markedly increase creatinine level. Administration of *Kleinhovia* sp. extract appears to comparatively lower the urea levels after DOX injection (III, IV, V) even though it is not considered statistically significant [Figure 2]. Although DOX-treated animals did not show a significant elevation of plasma creatinine, there was a trend of lower creatinine level in *Kleinhovia* sp.-treated Groups (III and V) compared to Group II.

Histopathological examination

In liver tissues [Figure 3], control rats showed normal architecture of hepatocytes. However, after 24 h of DOX i.p. injection (Group II), rat livers showed extensive degeneration of hepatocytes, hydropic changes, with signs of necrotic cells and vacuoles. *Kleinhovia* sp. treatments prior to DOX injection led to improved structures, which was dose dependent. In a lower dose (100 mg/kg), rat livers were shown to have some necrotic cells and vacuoles but it was not as extensive as Group II. Using higher doses (Group IV and V), administration of *Kleinhovia* sp. extract led to a significant improvement of tissue architecture although signs of congestion were still apparent in both groups.

In cardiac tissue, treatment with DOX only (Group II) caused significant structural changes in myocardium of rats, including loss of cross striation and eosinophilic cytoplasm, which indicate progression of myocardial infarction. Scattered pyknotic nuclei

were also evident in myocardium of rats in Group II. Eosinophilic area and loss of cross striation were still apparent in rats treated with *Kleinhovia* sp. extract 100 mg/kg (Group III), as well as in rats treated with 500 mg/kg (Group V) in a less rigorous manner. Meanwhile, treatment with 250 mg/kg *Kleinhovia* sp. extract (Group IV) showed significantly improved myocardial structure [Figure 4].

In renal tissue, control rats showed a normal architecture of glomerulus and tubules. In rats treated with DOX only, the structure of renal tubules was significantly altered, which mostly showed swelling, hydropic degeneration of tubules, and vacuolization. Necrotic changes and pyknotic cells were also evident in Group II. In *Kleinhovia* sp.-treated rats with dose of 100 mg/kg (Group III), the renal tubules still showed hydropic degeneration and vacuolization, with hemorrhagic glomerulus. With higher doses of extract (250 mg/kg and 500 mg/kg), less pathological changes were found in renal tubules, but glomerular hemorrhage is still evident [Figure 5].

DISCUSSION

Regardless of its potent chemotherapy action, DOX administration often leads to complications in patients with cancer. Due to its cardiotoxicity, the prevalence of cardiovascular diseases in patients with breast cancer significantly increases with DOX chemotherapy.^[11] This necessitates the search of cytoprotective agents that may halt DOX toxicity. Toxic effects of DOX are triggered from its conversion to semiquinone form, which

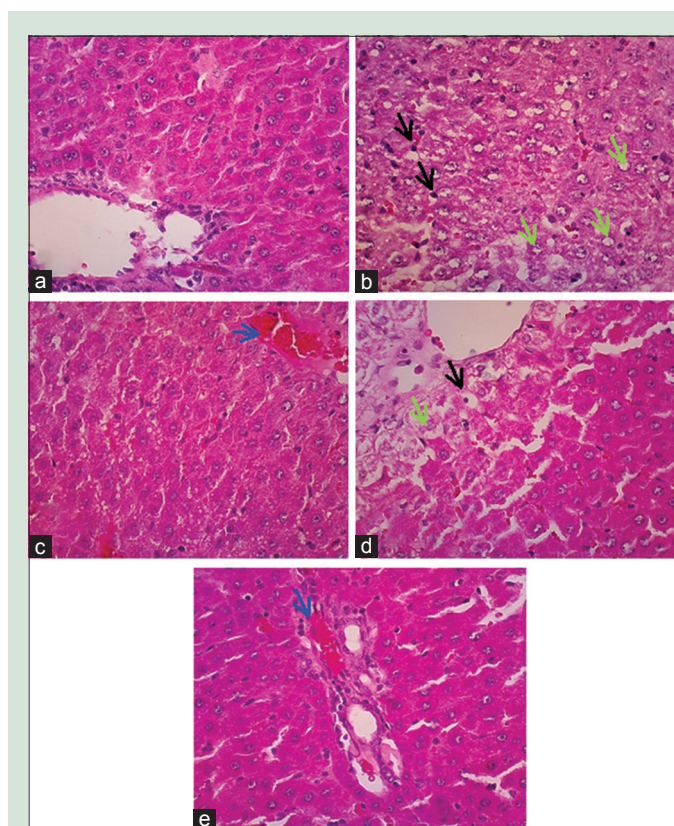


Figure 3: Representative of liver tissues in controls, rats treated with doxorubicin only or with *Kleinhovia* sp. controls (a) had normal liver structure, Group II (b) shows extensive damage with necrotic cells (black arrow), degeneration and vacuoles (green arrow). Group III with *Kleinhovia* sp. 100 mg/kg (c) shows slightly damaged area, necrotic cells, and vacuoles. Group IV with *Kleinhovia* sp. 250 mg/kg (d) shows improved structures with congested vessel (black arrow). Group V with *Kleinhovia* sp. 500 mg/kg (e) had congested vessels but hepatic tissue mostly improved

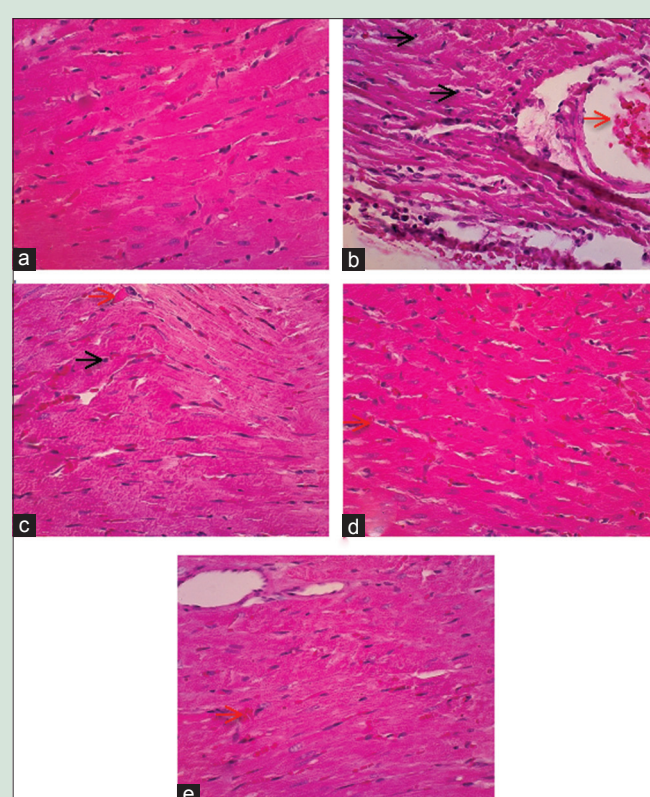


Figure 4: Representative of myocardial tissue in controls, rats treated with doxorubicin only or with *Kleinhovia* sp. Group control (a) shows a normal myocardial architecture. Group II with doxorubicin only (b) had loss of cross striation, pyknotic cell (black arrow), and congestion (red arrow). Group III with *Kleinhovia* sp. 100 mg/kg (c) shows hemorrhage and pyknotic cells and loss of cross striation. Group IV with *Kleinhovia* sp. 250 mg/kg (d) shows near-normal myocardium. Group V with *Kleinhovia* sp. 500 mg/kg (e) shows absence of cross striation and hemorrhage (red arrow)

is further converted into free radical metabolites (C7-radical DOX).^[12] These metabolites, in turn, increase the formation of reactive oxygen species (ROS), which lead to oxidative stress^[13] and cellular toxicities.^[14] Some animal studies have been conducted to find better protection against DOX toxicity.^[15-18] Although many of them have shown promising results, only iron chelating agent, dexrazoxane, has been clinically approved as an adjuvant therapy to reduce DOX-induced cardiotoxicity so far.^[19] This present study shows that *Kleinhovia sp* leaf extract may also provide benefits to alleviate hepatotoxicity and cardiotoxicity of DOX although its benefit in DOX-induced nephrotoxicity was not as evident. In this study, a single injection of DOX (25 mg/kg) triggered a significant elevation of the liver and cardiac cell biomarkers, indicating its deleterious effects on those organs' function. Toxic effect of DOX is associated with increased formation of ROS, profuse release of pro-inflammatory cytokines and inflammation, and induction of apoptotic and necrotic changes in these organs.^[20,21] The use of *Kleinhovia sp.* (especially in dose of 250 mg/kg) prior to DOX injection led to reduced ALT, AST, and CK-MB compared to those of DOX-treated rats [Figure 2], indicating a superior protection toward liver and cardiac cells. Interestingly, analysis for renal biomarkers did not show a significant difference between DOX-only and *Kleinhovia sp.*-pretreated animals.

Histopathological examination reveals that DOX induces extensive damage to the liver, heart, and renal structures. This present study showed

that administration of higher doses of *Kleinhovia sp.* (250 and 500 mg/kg) before DOX injection could maintain hepatocyte structures with few histological changes, including congestion. Congestion might be, in part, a result of anesthetic use during anesthesia prior to euthanasia. In cardiac tissue, a near-normal histological appearance was shown in animals treated with 250 mg/kg *Kleinhovia sp.* Although *Kleinhovia sp.* extract might also improve renal tubule integrity [Figure 5], yet the glomerulus still shows signs of hemorrhage in *Kleinhovia sp.*-treated animals at any given dose. This may suggest that protective effect of *Kleinhovia sp.* against DOX toxicity is limited in renal tissue.

Leaves of *Kleinhovia sp.* from family of Sterculiaceae are known to contain flavonoids such as kaempferol, quercetin, and rutin.^[22] Further studies have isolated a range of bioactive compounds from this plant. These include kaempferol 3-O-b-D-glucoside and eleuthero,^[7] cycloartane triterpenoids including gardenolic acid B,^[5] and Kleinhospitines A-D.^[6] More recently, Mo *et al.* isolated two new cycloartane triterpenoids form of *K. hospita*, namely (23R)-21,23,23,27-diepoxychoartan-1,24-diene-3,27-dione and (3 α)-(α -L-arabinopyranosyloxy)-1 α -hydroxy-23-oxocycloartan-28-oic acid.^[23] Most of these bioactive compounds showed potent antioxidant activities and cytotoxicity against cancerous cells,^[4,7,23] as well as promising hepatoprotective effects.^[5] Currently, extracts of *K. hospita* are mostly used in Indonesian communities for treating hepatitis and hepatotoxicity, but its potential use as an antitumor agent has now gained more attention.^[4,5]

CONCLUSION

Kleinhovia sp. extract has potential to reduce DOX-induced liver and cardiac damage, especially in the dose of 250 mg/kg. This contention is also supported by improved hepatic and cardiac tissue architecture following *Kleinhovia sp.* extract administration. Regardless of some improvement in renal tubule histology, administration of *Kleinhovia sp.* ethanolic extract has a limited impact on reducing renal biomarkers.

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Conflicts of interest

There are no conflicts of interest.

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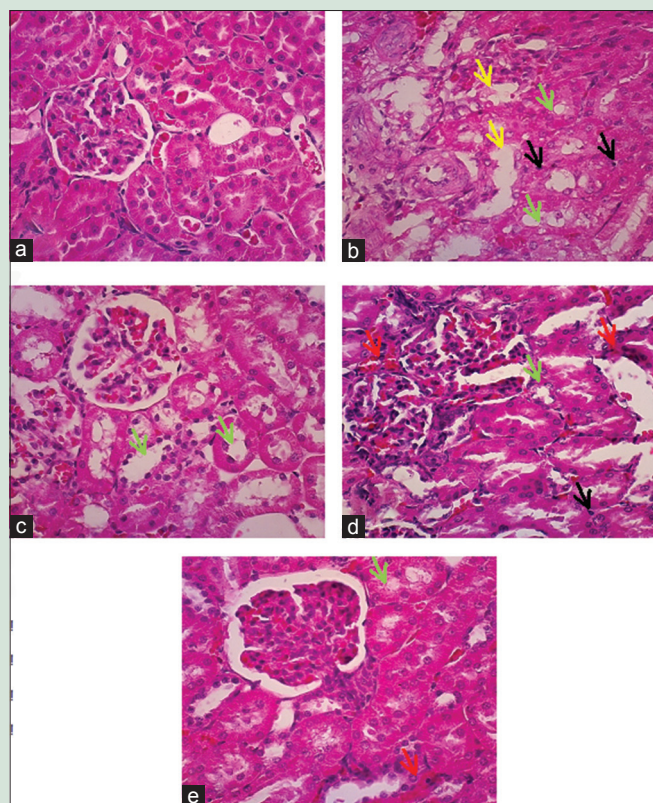


Figure 5: Representative of renal histology in controls, rats treated with doxorubicin only or with *Kleinhovia sp.* Group I (a) shows normal tubule architecture. Group II (b) shows extensive damage with tubule hydropic degeneration (yellow arrow), vacuolization (green arrow), and necrotic cells (black arrows). Group III with *Kleinhovia sp.* 100 mg/kg (c) shows hemorrhagic glomerulus with dilated tubules. Group IV with *Kleinhovia sp.* 250 mg/kg (d) shows improved tubule structure with hemorrhagic glomerulus. Group V with *Kleinhovia sp.* 500 mg/kg (e) shows near-normal renal tubules with hemorrhage (red arrow)

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