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Original Article

Curcuma zedoaria Extract as a Potential Protective Agent against Doxorubicin-Induced Toxicities in Rats

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Abstract

DOX therapy as an anticancer agent by incorporation of either physical treatments or antioxidant supplementation. *Curcuma zedoaria* (Rosc) (family Zingiberaceae) is an indigenous plant with antioxidant and anticarcinogenic activity. This study investigates the potential protective activities of an ethanol extract from *Curcuma zedoaria* (CZE) against DOX toxicities on non-target organs. A total of 50 female Wistar rats were divided into five groups: control group (G1), DOX only (G2), CZE 350 mg/kg + DOX (G3), CZE 525 mg/kg + DOX (G4), and Vit E + DOX (G5). Daily administration of CZE was given intra-gastric for 30 days, while 5 mg/kg DOX was injected concomitantly each on days 7, 14, and 21. The results of blood biochemical analysis indicated that administration of either the single or multiple doses of DOX (G2) caused significant elevation in the blood levels of alanine aminotransferase (ALT), aminotransferase (AST), creatinine, and CK-MB, indicating that the tissue damages occurred in the liver, kidney, and heart. Treatments with CZE demonstrated that ALT, AST, creatinine, and CK-MB levels remained similar to baseline levels, or no elevation was observed in all groups (G3 and G4). The oral administration of CZE in doses of 350 mg/kg and 525 mg/kg also decreased the AST/ALT ratio independent of the dose given. However, 350 mg/kg CZE indicated a faster response to protecting activities than other treatments. In conclusion, administration of a *Curcuma zedoaria* (Berg.) Roscoe ethanol extract, particularly in the 350 mg/kg dose, can potentially prevent or reduce DOX's toxicities in the liver, kidney, and cardiac cells.

Keywords: *Curcuma zedoaria*; Doxorubicin; Protective; Toxicities.

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1. Introduction

Doxorubicin (DOX) is an anthracycline antibiotic originally discovered from algae, *Streptomyces peucetius var. caesius [1]*. DOX is used as a broad-spectrum chemotherapeutic

agent in various cases of hematological malignancies and some cases of solid tumors, including carcinoma in ovarian, thyroid, breast, and prostate [2, 3]. Nevertheless, the use of DOX has been associated with the risk of cytotoxicity in non-target cells, leading to damage to some organs, such as the liver, kidneys, brain, and, most importantly, the heart [4-6].

Several studies mentioned the role of oxidative stress as the response to an intravenous administration of DOX. The production of reactive oxygen species (ROS) by DOX causes the death of targeted cancer cells and a systemic injury affecting other vital organs [7, 8]. The oxidative stress by ROS stimulation potentially initiates or promotes molecular oxidative damage to affected membrane lipids and DNA [9]. Therefore, it is necessary to identify effective treatments against the risk of oxidative stress to maintain or enhance the complementary use of DOX therapy as an anticancer agent by incorporating physical treatments or antioxidant supplementation [10, 11].

Curcuma zedoaria (Rosc) (family Zingiberaceae) is an indigenous plant with antioxidant and anticarcinogenic activity. The rhizome has been empirically used as an antimicrobial, antiallergic, anti-inflammatory, and hepatoprotective [12-14]. *Curcuma zedoaria* is a food supplement to prevent cardiovascular damage [15]. Oral administration of a *Curcuma zedoaria* (Rosc) ethanol extract (CZE) had a beneficial effect in preventing the risk of liver damage caused by cigarette smoke exposure in rats [16,17]. Despite its potency, based on our understanding to date, no research has been conducted on the protective effect of CZE on DOX-induced toxicities in some organs. Therefore, this experiment was designed to investigate the protective effect of CZE on the liver, kidney, and heart toxicities caused by DOX in rat models.

2. Materials and Methods

2.1. Preparation of the Curcuma zedoaria Extract (CZE)

Fresh rhizomes of *Curcuma zedoaria* were obtained from Makassar, South Sulawesi, and authenticated in the Laboratory of Biology, Faculty of Mathematics and Natural Sciences, Makassar State University. The samples were cut into small pieces, dried using a vacuum dryer at 45℃ for 24 h, and passed through an 18-siever. The coarse powder was subjected to the extraction process with ethanol 70% v/v as the solvent. Curcuma zedoaria (CZE) extract was prepared as described previously [16]. CZE was characterized micro-and macroscopically based on the standard of the 2008 Herbal Pharmacopoeia of Indonesia. After preparation, CZE was packed in an air-tight container and stored at 5℃ ± 1℃ for further analysis or use.

2.2. Drugs and reagents

Doxorubicin (DOX) was purchased from a certified Pharmaceutical Distributor. Diagnostic kits for biochemical analysis were supplied from Human Diagnostic Worldwide® (Germany).

2.3. Animals and experimental protocols

Seven days before the experiment, female Wistar rats (Rattus norvegicus) aged 8–10 weeks were acclimatized to environmental and experimental laboratory conditions with a 12 h light-dark cycle at 25℃ ± 3℃, and food and water were provided

ad libitum. To identify the healthy animals, the blood samples were withdrawn via a lateral vein for pre-treatment biochemical analysis. The method used in this experiment was approved by the ethical committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, with protocol number 306/UN4.6.4.5.31/PP36/2021.

2.4. DOX injection

Kemodoxin® (DOX) by PT. Otto Pharmaceutical Industries was purchased from a Pharmacy. The drugs were reconstituted in sterile water before use. The concentration of DOX was adjusted to contain the required dose in 1 ml and injected intraperitoneally (i.p.) in each animal, following [18].

2.5. The administration of Curcuma zedoaria

The dose of CZE used in this study was chosen based on the effective doses of CZE as an antioxidant in other studies [16,17]. Fifty rats were randomly divided into five groups (n = 10): Control group (G1); 15 mg/kg DOX group (G2); 350 mg/kg CZE + DOX group (G3); 525 mg/kg CZE + DOX group (G4); and 150 mg/kg Vit E + DOX group (G5). Daily administration of 1% NaCMC alone (G1), CZE in 1% NaCMC (G3 and G4), or Vit E in 1% NaCMC (G5) were performed intragastrically for 30 consecutive days. Organ damages induced by DOX (G2–G5) or normal saline (G1) in a dose of each 5 mg/kg were injected on the 7th, 14th, and 21st days of treatment.

2.6. Enzyme analysis

Rat weights were recorded weekly, and blood collection for biochemistry analysis was performed 24 hours after treatments on the 7th and 30th days. Blood withdrawn from the lateral tail vein was centrifuged at 1,500 rpm for 10 min. Blood serum levels were determined using Humalyzer 3500 (Human*) to investigate the effect of CZE through analysis of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes as the parameter of liver function, Kreatinin as a parameter of renal function, and creatine kinase-MB (CK-MB) as biomarkers of myocardial injury. Biomarker measurements of AST, ALT, creatinine, and CK-MB were analyzed using Humalyzer 3500 (Human®) instrumentation based on the diagnostic kit instructions.

2.7. Statistical analysis

Data are reported as mean ± standard deviation. Normal distribution was statistically analyzed with the Kolmogorov–Smirnov test. Normally distributed data were analyzed using a one-way analysis of variance and then Tukey's significant-difference posthoc test. $P < 0.05$ was considered to indicate a significant difference.

3. Results and Discussion

The viscous, semi-solid, brownish extract with a specific odor of *C. zedoaria* rhizome was obtained with a yield value of dried simplicia ranging from 5% to 8%. The characteristics of the ethanol extract of *C. zedoaria* obtained were properly suitable as a standard extract described in Herbal Pharmacopoeia of Indonesia [19].

During the *in vivo* experiment, each rat's general visual observation and body weight measurements were determined daily.

It was demonstrated that the treatment group (G2–G5) resulted in reduced body weights compared with those in the control group (G1). However, there was no significant difference in the body weight of the CZE-DOX group (G3 and G4) or Vit E + DOX group (G5) compared with that of the DOX group (G2) (data not presented). Determination of the biochemistry level from blood samples was performed on day 0 as the normal baseline, day 8 to identify acute alterations, and day 31 to assess sub-chronic effects.

Vitamin E was used as a control due to its properties as an antioxidant agent. Vitamin E is an organic micronutrient that dissolves in fats and contributes to maintaining human health. Primarily, it acts as a scavenger for free radicals, safeguarding biological membranes from damage caused by lipid peroxidation. In addition to its traditional role as an antioxidant, vitamin E regulates enzyme activities, signaling pathways, and the expression of genes and proteins. Numerous cellular responses, including inflammation, cell growth, programmed cell death, and lipid balance, have been influenced by vitamin E; this was observed through lab and animal experiments [20].

ALT and AST serum levels have been widely used clinically as the Liver Function Test parameters for diagnosing liver damage. Measurement of liver enzymes on day 0 indicated that the average levels of AST and ALT were in the range of reference values for female rats [21, 22], meaning that the entire animal used had a healthy liver function.

As illustrated in **Figure 1**, DOX alone (G2), both at single-dose and multiple-dose administrations, induced significant increases in serum ALT or AST levels compared with those in the control group ($P<0.05$), and these increases were effectively prevented or reduced by treatment with CZE. Single-dose administration was giving the substance once at a specific time point. It aims to observe the substance's immediate effects or short-term response. This approach is useful when the research question revolves around the acute impact of the substance and its quick effects. Meanwhile, multiple-dose administration was giving the substance to the animal repeatedly over a certain time. It aims to simulate a more prolonged or chronic exposure, mimicking a situation closer to how a drug might be administered to an animal over an extended treatment regimen. Multiple injections can provide insights into the cumulative and long-term effects of the substance.

Llesuy and Arnaiz found that the DOX toxicity mechanism is involved in the process of antioxidant reduction [23]. After six days of treatments with CZE followed by a single DOX injection to mimic the acute response of the liver, the levels of AST and ALT rapidly rose almost two-fold higher than the baseline level. Administration of CZE (G3 and G4) 6 days before DOX induction maintained the level of AST and ALT enzymes similar to the baseline level and the control group (G1). Moreover, simultaneous administration of CZE for 30 days on rats induced by multiple doses of DOX also indicated a statistically significant difference between the experimental groups (G3 and G4) and negative controls (G2) using liver enzyme levels. Although changes related to liver cell damage were more prominent after multiple-dose DOX administration, there was no significant difference between the healthy control (G1) and groups given CZE biochemically (G3 and G4).

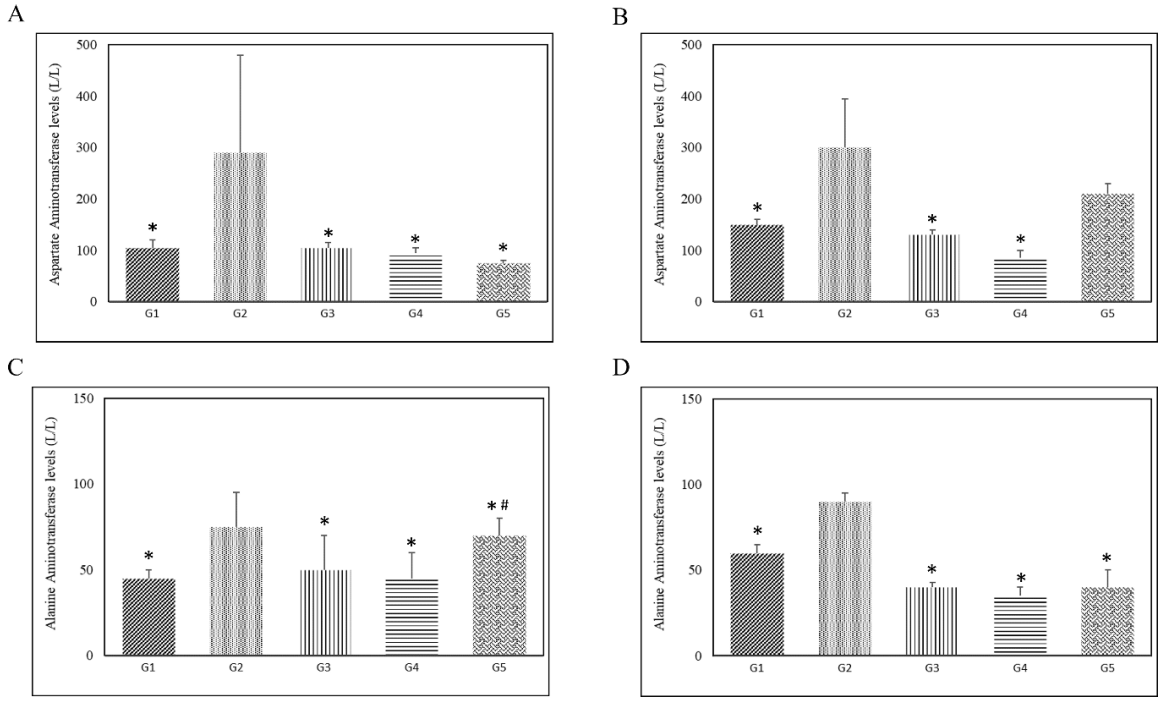

Figure 1. Results of liver function test analysis in the serum of rats after DOX-induced hepatotoxicity; Levels of AST with single-dose injection (A) or multiple-dose injections (B). The levels of ALT with single-dose injection (C) or multiple-dose injections (D). Values are presented as the mean ± standard deviation. G1: controls, G2: 15 mg/kg doxorubicin, G3: 350 mg/kg *CZE* + doxorubicin, G4: 525 mg/kg *CZE* + doxorubicin, G5: *150* mg/kg *Vit E* + doxorubicin. (gray box) Indicates baseline levels. * $P < 0.05$ compared with the doxorubicin group (G2). # $P < 0.05$ compared with the control group (G1).

Notably, it was observed that the high level of ALT in the DOX + Vit. E acute group (**Figure 1C**) declined after longer Vitamin E administration (**Figure 1D**). A hepatoprotective effect in the group that received Vitamin E was similarly observed in other studies that used DOX to induce oxidative stress in rats' livers [24,25]. Therefore, relatively similar data that showed declining AST and ALT levels in the groups treated with 350 mg/kg (G3) or 525 mg/kg (G4) CZE could be associated with the antioxidant properties of CZE to prevent the hepatotoxicity effect of DOX. These findings suggest that DOX-induced liver damage could be efficiently reversed by CZE or vitamin E administration, yet this recovery was more prominent in the group given CZE. This finding followed other studies that found that the ethanol extract of Zingiberaceae had free radical scavenger activity to prevent damage to hepatocytes but relatively had no toxic effect on liver cells [26,27]. The hepatoprotective effect of *Curcuma zedoaria* was supported by the antioxidant activity of curcumin and other essential oils as active compounds that contain monoterpenes and sesquiterpenes (furanogermone) [14].

The creatinine level was measured from the serum of the animal model to investigate the renal cell damage caused by DOX nephrotoxicity (**Figure 2**). Since creatinine is the by-product of creatine phosphate hydrolysis in a skeletal muscle that entered the blood and was cleared entirely at

a constant rate by the glomerulus, elevation of this endogenous marker in the blood is commonly used to estimate renal function.

After injection of single and multiple doses of DOX, the trend of the blood creatinine level increased with a higher appearance shown later mentioned. Even though the difference was not markedly significant in the statistical analysis, administration of CZE at 350 mg/kg (G3) and 525 mg/kg (G4) showed a lower creatinine level compared to DOX alone group (G2). Therefore, treatment of CZE preceding or following DOX injection can effectively prevent DOX nephrotoxicity.

Aspartate aminotransferase enzyme or AST is found in the liver, heart, skeletal *muscle*, kidneys, brain, and red blood cells; thus, its elevation may be found during not only liver injury but also heart damage, hypoxia or extensive trauma [28]. The result indicated that AST levels in Group II significantly elevated either 24 h after a single dose of DOX injection or after multiple-dose treatments, indicating potential damage in liver and heart cells, correspondingly. By contrast, treatments with CZE demonstrated hepatoprotective and cardioprotective effects shown by similar AST with baseline levels or no AST elevation in all groups independent of the dose given (Figures 1A and 1B). These data are also supported by CK-MB analysis and evaluation of the AST/ALT ratio.

Data from this study described a high ratio found in all groups, including those that received 1% NaCMC and 0.9% NaCl injection (**Table 1**). Several studies described a higher ratio of AST/ALT (>1) correlates with a greater probability of hepatic fibrosis associated with cardiovascular disease through different pathogeneses, including oxidative stress response. AST alone is not considered a specific marker for myocardial damage.

However, since AST is released mostly from the myocardium and the liver tissues, whereas ALT is only released from the liver, more severe myocardial pathology would lead to an anticipated increase in the AST/ALT ratio. Therefore, the aspartate aminotransferase-to-alanine aminotransferase ratio (AST/ALT) is recently considered a new predictor associated with functional severity in chronic heart failure [29, 30].

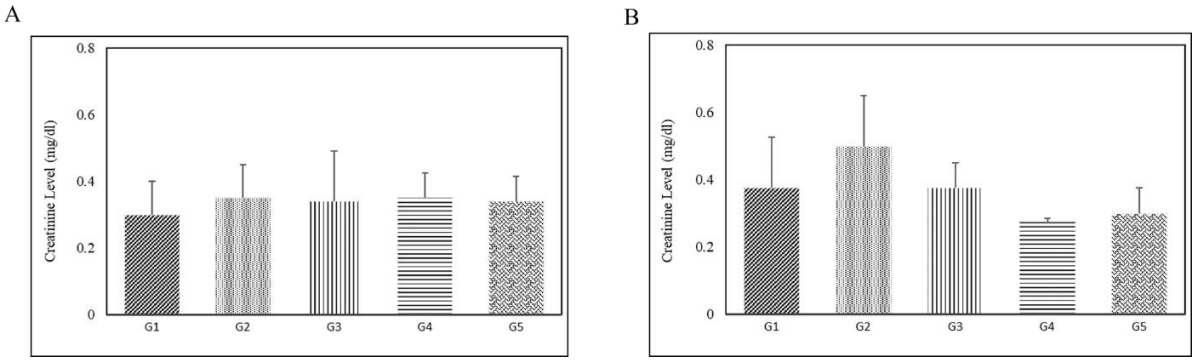

Figure 2. Results of creatinine levels in the serum of rats after DOX-induced hepatotoxicity with single-dose injection (A) or multiple-dose injections (B). Values are presented as mean ± standard deviation. G1: controls, G2: 15 mg/kg doxorubicin, G3: 350 mg/kg *CZE* + doxorubicin, G4: 525 mg/kg *CZE* + doxorubicin, G5: *150* mg/kg *Vit. E* + doxorubicin. (gray box) Indicates baseline levels.

Table 1: The level of blood biomarkers for rats' liver function tests after 30 days of treatments using the ethanol extract of *Curcuma zedoaria* against multiple doses of doxorubicin-induced organ-damaged.

Treatment Groups	Mean ± SD		
	AST (U/L)	ALT (U/L)	AST/ALT Ratio
Control (G1)	152.4000 ± 15.51	58.4266 ± 7.31	2.6084 ± 0.39
15 mg/kg DOX (G2)	345.2000 ± 11.88	95.7333 ± 6.28	3.6058 ± 0.34
350 mg/kg CZE + 15 mg/kg DOX (G3)	124.9667 ± 10.76	37.8933 ± 2.23	3.2979 ± 0.48
525 mg/kg CZE + 15 mg/kg DOX (G4)	72.2650 ± 21.33	32.1050 ± 5.98	2.2509 ± 0.62
150 mg/kg Vit. E + 15 mg/kg DOX (G5)	215.7750 ± 42.28	42.6200 ±.8.60	5.0628 ± 1.82

AST/ALT ratio supported biomarker analysis of CK-MB levels, as illustrated in **Figure 3**. After DOX injection at either single or multiple doses, the CK-MB level increased rapidly in the DOX-only group (G2). With doses of 350 and 525 mg/kg, CZE administration effectively reduced CK-MB level, but dose 350 mg/kg appeared superior, providing a faster response of protection against DOX-induced cardiac toxicity. These data greatly supported the protective role of herbals' occupied antioxidant properties in preventing cardiac toxicity induced by single or multiple doses of doxorubicin injections [6, 11, 26]. Multiple mechanisms have been suggested to explain the cardiotoxic effects and heart failure induced by Doxorubicin (Dox). The cardiac issues associated with Dox stem directly from heightened oxidative stress within heart tissue. Elevated oxidative stress triggers the generation of reactive oxygen species (ROS), which inflict harm on the heart muscles. Diminished levels of antioxidants and sulphhydryl groups exacerbate this. Additionally, Dox-triggered cardiotoxicity involves disrupted levels of calcium ions, resulting in apoptosis. This process occurs in cardiomyocytes and endothelial cells, involving the activation of caspases [31]. In this discussion, we have comprehensively explored the molecular mechanisms contributing to Dox-induced cardiotoxicity. The chief concern is the generation of free radicals, which cause damage to cardiac muscle cells following the administration of Dox [32]. Dox-related cardiotoxicity manifests via increased production of ROS and lipid peroxidation within cardiac tissues [33,34]. Aglycones and iron complexes of anthracyclines prompt ROS production [35]. Over the past three decades, numerous molecular mechanisms have been proposed to explain ROS generation and its detrimental impact on heart tissue.

These mechanisms involve various enzymes such as nitric oxide synthase (NOS), NADPH oxidase (NOX), mitochondrial-dependent ROS generation, and more, all of which trigger oxidative stress [31]. We will discuss these mechanisms below.

Doxorubicin binds to the enzyme endothelial nitric oxide synthase (eNOS) reductase, inducing the formation of Dox semiquinone radicals. These radicals convert free oxygen into superoxide (O-2), a free radical. This reactive transformation follows an enzymatic one-electron reduction reaction, adversely affecting the heart. Dox completes this one-electron reduction reaction in the presence of flavoenzymes like NADPH-cytochrome P450 reductase and mitochondrial NADH dehydrogenase [35].

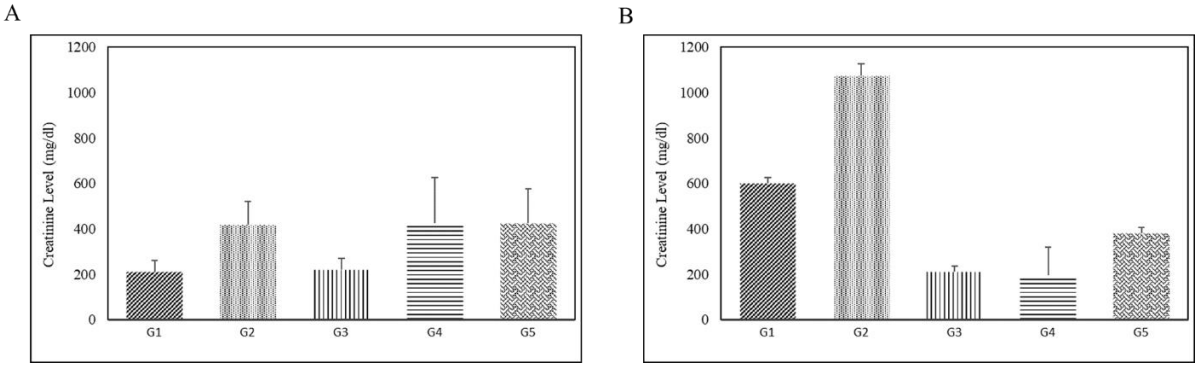

Figure 3. Results of creatinine kinases-MB levels in the serum of rats after DOX-induced hepatotoxicity with single-dose injection (A) or multiple-dose injections (B). Values are presented as mean ± standard deviation. G1: controls, G2: 15 mg/kg doxorubicin, G3: 350 mg/kg *CZE* + doxorubicin, G4: 525 mg/kg *CZE* + doxorubicin, G5: *150* mg/kg *Vit. E* + doxorubicin. (gray box) Indicates baseline levels. * $P < 0.05$ compared with the doxorubicin group (G2). # $P < 0.05$ compared with the control group (G1).

The binding of the drug to the eNOS reductase enzyme disrupts the balance between superoxide free radicals and nitric oxide levels. Nitric oxide levels decrease while superoxide levels increase, leading to cardiotoxicity [36]. A study demonstrated that administering Dox to bovine aortic endothelial cells increases eNOS mRNA and protein levels, activating redox stimulation and resulting in apoptosis [37]. Interestingly, antisense eNOS mRNA reduced the activation of caspase-3 activity, implying a cardioprotective effect against Dox-induced cardiotoxicity. Similarly, transgenic eNOS mice exhibited heightened Dox-induced cardiac ROS production, whereas knockout mice showed decreased cardiac ROS levels [38].

4. Conclusion

The oral administration of a *Curcuma zedoaria* (Berg.) Roscoe ethanol extract in doses of 350 and 525 mg/kg BW reduced ALT, AST, creatinine, and CK-MB serum levels and decreased the AST/ALT ratio in rats induced by doxorubicin. Therefore, we conclude that the *Curcuma zedoaria* (Berg.) Roscoe ethanol extract has the potential to prevent or reduce DOX-induced liver, kidney, and cardiac toxicities, particularly in the dose of 350 mg/kg BW.

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Conflict of interest

The authors declare to have no conflict of interest.

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