

The potential protective effects of malacca (*Phyllanthus emblica* L.) extract against doxorubicin-induced cardiotoxicity in male Wistar rats

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Abstract. Doxorubicin as a chemotherapy agent is most widely used in cancer treatment. Long-term use at a predetermined dose has a side effect, namely cardiotoxicity. Doxorubicin-induced cardiotoxicity is considered to be caused by reactive oxygen species (ROS), which is also characterized by increasing CK-MB enzyme levels in the blood. To reduce the impact of doxorubicin-induced toxicity, a study was conducted on natural antioxidant sources with cardioprotective capabilities *in vivo*. *Phyllanthus emblica* L. fruit was used as a sample for a natural source of antioxidants extracted using ethanol. Then performed a phytochemical screening of secondary metabolites contained in it. This extract was administered orally in various doses to the experimental animal Wistar rats and the induced doxorubicin to these animals. The CK-MB enzyme levels were measured, and the heart organ histopathology test was performed. The results of this study indicate that *P. emblica* L. fruit extract contains alkaloids, tannins, flavonoids, terpenoids, phenolics, and triterpenoids compounds. Extract treatment at a 400 mg/kg BW dose showed the best reduction in CK-MB levels with great improvements in regular arrangement and shape of myocardial muscle cells of cardiac tissue. The sample extract at a 400 mg/kg BW dose showed remarkably decreasing of CK-MB great improvements of heart tissue on doxorubicin-induced cardiotoxicity. This study showed the potential protective effect of *P. emblica* L. against doxorubicin-induced cardiotoxicity.

Keywords: *Phyllanthus emblica* L.; Doxorubicin cardiotoxicity; CK-MB

INTRODUCTION

Doxorubicin, a member of the anthracycline family, is a chemotherapeutic agent commonly used to treat hematologic malignancies and solid tumors (leukemia, lymphoma, osteosarcoma, lung cancer, breast cancer, etc.) [1], [2]. Many studies found that prolonged exposure to doxorubicin in patients can lead to hypertension, left ventricular disease, and heart failure [2]–[5]. If traced history, it is generally caused by dose-dependent, cumulative, and progressive [6], [7]. This incident causes people to become aware of cardiotoxicity –induced by doxorubicin.

The cardiotoxicity caused by doxorubicin has long been studied by researchers [6], [8]–[10]. Volkova & Russell (2011) explained that patients treated with doxorubicin tend to give

clinical signs and congestive heart failure symptoms. There is a decrease in the left ventricular ejection fraction (LVEF) function caused by an accumulation of doxorubicin doses. The escalating cardiotoxicity typically occurs after the completion of doxorubicin treatment. It can become evident in weeks after treatment (early-onset chronic cardiotoxicity) or several years after completion of chemotherapy (late-onset chronic cardiotoxicity). The mechanism of doxorubicin cardiotoxicity is still remaining unclear. However, a number studies found that reactive oxygen species (ROS) is one of main reasons for doxorubicin-induced cardiotoxicity [8].

Bioactive compounds of secondary metabolites have been believed to be able to againsts ROS [11]. Therefore this approach to reducing heart failure from doxorubicin can be carried out through secondary metabolite compounds in plant extracts. Xiong 2018 found that the berberine compound (isoquinoline alkaloid) extracted from *Coptis chinensis* effectively reduced the effect of DOX-induced heart tissue free radical injury in mice. Berberine worked by lowering the serum creatine kinase isoenzyme

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(CK-MB) level in the DOX group. Another alkaloid compound, oxymatrine extracted from *Sophora flavescens*, also has a cardioprotective effect on DOX-induced rats [12]. Furthermore, the ethanolic extract of *Curcuma Longa* was also found to reduce cardiac biomarker (CK-MB) levels [13].

Creatine kinase myocardial band (CK-MB) is an enzyme found in the cardiac muscle fraction. CK-MB is indicated as serum cardiac biomarker for early clearance of myocardial infarction [14], [15]. This enzyme has been a traditional gold standard for acute myocardial infarction (AMI) [14], [16]. Along with other serum cardiac markers, the CK-MB level fraction is evaluated for myocardial reinfarction diagnosis [15].

Since the secondary metabolite from plant extracts containing several bioactive compounds has been shown to reduce CK-MB levels, this study examines the cardioprotective ability of Malacca (*Phyllanthus emblica* L.) fruit extracts. This research was conducted *in vivo* on male rats that had been induced by doxorubicin. The ability of *P. emblica* L. fruit extract was observed through CK-MB levels in serum samples and histopathological studies.

METHODOLOGY

Materials

Phyllanthus emblica L. fruits were obtained from local market at Medan city and were determined at Laboratory of Plant Taxonomy, Biology Department, Mathematics and Natural Science Faculty, Universitas Sumatera Utara. *Mus musculus* mice male was collected from medicine faculty of Universitas Prima Indonesia. The chemical materials were used potassium iodide, iron (III) chloride, acetic anhydride, iodine, magnesium powder, bismuth (III) nitrate, eosin, and hematoxylin solution were purchased from Sigma Aldrich, USA. CK-MB reagen kit was purchased from PROLINE, Indonesia. Doxorubin-HCl was obtained from KalbeMed, Indonesia. Hydrochloric acid, phosphate buffer, amyl alcohol, sulfuric acid, and ethanol were purchased from Smartlab, Indonesia.

Methods

Sample extraction

Sample preparation and extraction followed the previous method with slight modification [17]–[19]. Fruit samples were dried at 50°C for two days and then finely ground, resulting in 800 g of Malacca fruit powder. The powder was soaked with 96% ethanol with a powder ratio and solvent 1: 3 (m/v). The mixture is shaken

for ± 48 hours at ± 200-250 rpm and filtered. The maceration process is carried out until the solvent was clear. Then, the extract was thickened using a rotary evaporator at a temperature of 50°C and extracted from *P. emblica* L. fruits.

Preliminary phytochemical analysis

The investigation of bioactive compound in *P. emblica* fruit extract had done qualitatively. Analysis was carried out by following standard method for phenols [20], terpenoids [21], alkaloids, tannins, flavonoids [22], and triterpenoids [23].

Experimental animals and CK-MB test

Male rats (Wistar) weighed 150-250 g were selected and they were acclimatized for a week. The acclimatization condition was 12 h light/dark cycles and was fed *ad libitum* with pellets and tap water at room temperature (under humid tropical conditions). This study was conducted under Health Research Ethics Committee of Universitas Prima Indonesia 028/KEPK/UNPRI/IX/2020 referring to WHO standard and 2016 Council for international Organizations of Medical Sciences (CIOMS) Guidelines. The dosage if the *P. emblica* L. extract was given followed previous study [24]. Those rats were divided into 5 groups consisted 5 rats for each group;

- Group 1 : No treatment for 14 days
- Group 2 : Doxorubicin 25 mg/kg BW through intraperitoneally for 3 days
- Group 3 : *P. emblica* L. 200 mg/kg BW was given orally for 14 days
- Group 4 : *P. emblica* L. 200 mg/kg BW was given orally for 14 days and Doxorubicin 25 mg/kg BW was given orally for 12th, 13th, 14th days
- Group 5 : *P. emblica* L. 400 mg/kg BW was given orally for 14 days and Doxorubicin 25 mg/kg BW was given orally for 12th, 13th, 14th days

The observation was done for 14 days. Three milliliter of blood was taken at day-15 after fasting for 18 h. After left for ± 20 min, the blood was centrifuged for 20 min at 3000 rpm and serum was obtained. Serum levels of CK-MB were measured using a commercial kit, Bio Majesty® PROLINE, and measured using a microplate reader at 340 nm.

Histopathology of rats cardiac tissue

In euthanized rats, the whole heart was surgically removed and washed with 0.9% NaCl solution to clean the remaining blood that had stuck. The color and surface texture of the rats' hearts were observed macroscopically and fixed in 10% formalin for 3 h [17], [19].

Preparations for cardiac histology were prepared according to previous work using Haematoxylin-Eosin stain [17], [19].

Statistical analysis

Values were expressed in terms of Mean $\pm$ SD. One-way ANOVA followed by 'Post Hoc t-tests' was done to determine the significance of inter-group differences. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical screenings

Phytochemical screening methods were widely used for the investigation of natural sources. Secondary metabolites contained various chemical compounds which playing an important role in the biological activity as defensive compounds [25] and protect the plant from biotic and abiotic stress [26]. The chemical compounds as a bioactive agent that can be used in herbs as traditional medication [27], [28]. Screening test were done qualitatively and presented in Table 1.

Table 1. The phytochemical present in *Phyllanthus emblica* L. (Malacca) fruit extracts

Compounds	Result	
	Fruit Extract	Observation
Alkaloids	(+)	Orange
Flavonoids	(+)	Yellow/Orange
Phenolics	(+)	Green/Blue/Black
Triterpenoids	(+)	Orange
Terpenoids	(+)	Purple
Tannins	(+)	Red/Orange Layer

+ = Present, - = Absent

Table 1 shows the result of phytochemical screening of *Phyllanthus emblica* L. (Malacca) fruit extracts with alkaloids, flavonoids,

phenolics, steroids/triterpenoids, terpenoids, and tannins as the part of secondary metabolites. *Phyllanthus emblica* seed has potential bioactive agents contained tannin and flavonoids with high antioxidant activity [29]. *P. emblica* fruits also contained phenolics that playing role as antioxidant and antiproliferative [30]. *P. emblica* (Malacca) bark-extract contained phenolic, flavonoids, and tannins compounds [30], [31]. The biological activity of *P. emblica* L. extracts reported as an anti-inflammatory [32], anti-microbial [33], antioxidant, anti-collagenase, and anti-elastase [34].

Body weight measurements

In this study, the different treatments were applied in different groups to observe the increase in rats' body weight. Table 2 shows the drastically increase of the rats' weight for all group treatments (normal, DOX-treated, fruit extract, DOX-induced with 200 mg/kg BW fruit extract, DOX-induced with 400 mg/kg BW fruit extract). Doxorubicin treatment showed the lowest increasing weight with 8.07 % of initial weight. DOX-induced with 400 mg/kg BW fruit extract group has the highest increase of body weight with 14.58 % from initial weight compare to the other treatments. This study stated that ethanol extract of *P. emblica* L. affects the increasing body weight of rats under doxorubicin-induced cardiotoxicity.

Biochemical results

The cardiotoxicity biomarkers measurements were conducted to evaluate the level of Creatine kinase-MB (CK-MB). CK-MB is the indicator of myocardial damage [35], [36]. The doxorubicin induces elevate the serum activities of CK, CK-MB, LDG and CT-I [37]. CK-MB is an enzyme in the myocardium that can use as an indicator of the existence and extent of myocytes injury. The DOX-induced free radical accelerate membrane peroxidation and disruption of cardiac myocytes that can increase CK-MB release in the serum [38].

Table 2. The body weight (BW) of rats for all of the group treatments

Group	Treatment	Initial BW (g)	Final BW (g)	Increasing of BW (%)
I	Normal	169.4 $\pm$ 6.68	190.8 $\pm$ 8.64	11.21
II	Doxorubicin	183.84 $\pm$ 4.19	199.98 $\pm$ 4.85	8.07
III	Fruit Extract (200 mg/kg)	171.04 $\pm$ 7.81	190.88 $\pm$ 8.63	10.39
IV	200 mg/kg BW Fruit Extract + DOX	184.48 $\pm$ 5.81	205.78 $\pm$ 6.22	10.35
V	400 mg/kg BW Fruit Extract + DOX	169.3 $\pm$ 6.26	198.2 $\pm$ 4.66	14.58

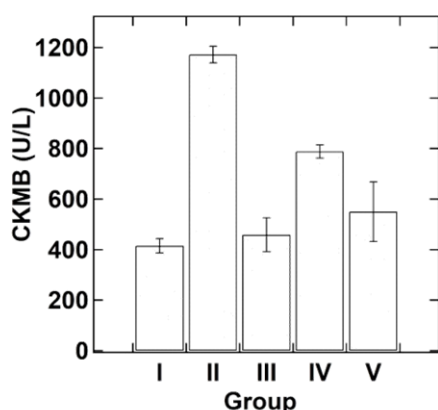


Figure 1. CK-MB values in different treatments on doxorubicin-induced rats. All values have been presented as mean $\pm$ standard error (n=5), $p < 0.05$. One-way ANOVA followed by 'Post Hoc t-tests' was done to determine the significance of inter-group differences. Group I; Normal, Group II; Doxorubicin-injected, Group III; Fruit Extract (200 mg/kg), Group IV; DOX-induced with 200 mg/kg BW Fruit Extract, Group V; DOX-induced with 400 mg/kg BW Fruit Extract.

In Figure 1, the normal group (Group I) shows the level of CK-MB at 415.6 ± 27.89 U/I as the doxorubicin treatment (Group II) increase the level of CK-MB at 1172.4 ± 32.54 U/I which the highest CK-MB levels from all of the treatments. As comparison, *P. emblica* L. fruit extract-treated group shows CK-MB level at 458.4 ± 66.76 U/I, similar to normal group. The CK-MB level activities reduce significantly in DOX-induced rats with *P. emblica* L. extract treatments compared to DOX-injected treatment group. Both 200 mg/kg BW dose and 400 mg/kg BW dose of fruit extract gave result enzyme level at 789 ± 26.50 U/ and at $550 \pm$

117.98 U/I, respectively. *P. emblica* L. extract at 400 mg/kg BW dose shows greatly decreasing level of CK-MB near to normal treatment in DOX-induced serum.

This result shows *P. emblica* L. fruit extract successfully decreases CK-MB concentration to prevent cardiotoxicity by doxorubicin. This finding agree with previous study by Sahyon & Al-Harbi (2020) found that extract of the heart of the *Phoenix dactylifera* tree also significant reduced CK-MB level in plasma compared to DOX-induced group. Hao et al. (2015) reported that alkaloid compound (Berberine) effectively reduces activity of CK-MB enzyme in rats induced by doxorubicin. Other study found that vanillic acid from phenolic family decrease serum levels of CK-MB in DOX-injected cardiotoxicity in rats [41].

Histopathology

The histological examination was investigating the *P. emblica* L. Extracts effect in doxorubicin-induced cardiotoxicity rats. This photomicrograph shows the histology of heart tissue in all treatments (Figure 2). The normal treatment shows normal arrangement of myocardial muscle fibers, the regularity shape of nucleus heart muscle cell, no visible cytoplasmic vacuolization, and no inflammatory cell infiltration of blood vessels. A single dose of DOX-Induced (Figure 2B) marked acute cardiotoxicity in rats after treatments. This was demonstrated by the increasing of CK-MB enzyme activities and confirmed by the histopathological changes in cardiac tissues. It is showed the irregularity in the arrangement and shape of myocardial cells. There are muscle cell vacuoles, visible infiltration of inflammatory cells in the blood vessels and interstitial edema.

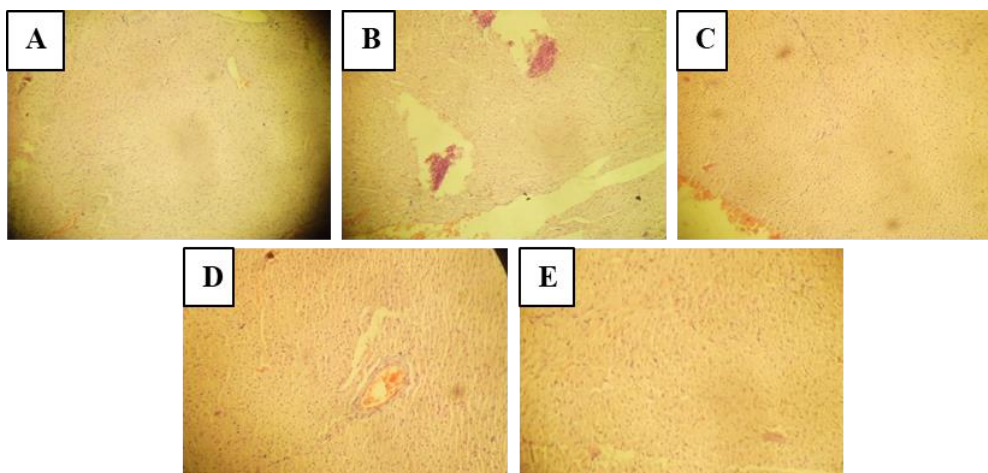


Figure 2. Histopathology of cardiac tissue of; A) normal treatment group, B) doxorubicin-treated group, C) *P. emblica* L. extract, D) Doxorubicin-induced + *P. emblica* L. extract 200 mg/kg BW, and E) Doxorubicin-induced + *P. emblica* L. extract 400 mg/kg BW with 400x magnification.

The treatments of *P. emblica* L. extract show the similarity with normal treatment's cardiac tissue, with slight inflammatory cell infiltration. Cardiac tissue of DOX-induced with 200 mg/kg BW and 400 mg/kg BW *P. emblica* L. extract (Figure 2D;2E) shows elevated structure of cardiac tissue, similar to normal and extract-treated group. *P. emblica* L. fruit extract at 400 mg/kg BW dose shows significant improvements in regular arrangement and shape of myocardial muscle cells, no visible cytoplasmic vacuole of cells, lower infiltration of inflammatory cells in the vessels blood and interstitial edema reducing.

DOX-Induced cardiotoxicity and inflammation were known with several mechanism such as free oxygen radicals level, alteration of myocardial cell membrane permeability due to lipid peroxidation, mitochondrial oxidative phosphorylation, and electrolyte contents [41]. Doxorubicin induces the cardiotoxicity by converting semiquinone form in the cardiac tissue and leads to the production of free radicals known as ROS, which accelerate the oxidative stress [42], [43]. Doxorubicin speeds up the free radical production such as: superoxide, hydroxyl radicals, and hydrogen peroxide which able to react with lipid causing lipid peroxidation [44]. Free radical such as peroxy alkoxy, and aldehyde were produced by lipid peroxidation resulting the cell damage and releasing marker enzyme [45]. The other mechanism is forming an anthracycline-iron free radical complex that will react with hydrogen peroxide producing radical [43]. Doxorubicin increased mitochondrial fragmentation followed by ROS generation and apoptosis [46]. The oxidative stress factor increases intracellular calcium and accelerates lipid peroxidation, which can damage the cell membrane and the other components [42].

Antioxidant well is known as the agent to decrease the doxorubicin-induced cardiotoxicity by eliminating free radicals [47]. The antioxidant activity of natural extract scavenging free radicals and inhibiting lipid peroxidation as the protective effect on doxorubicin-induced cardiotoxicity [47]–[49]. The previous study by Karimi et al. (2005)[48] reported lycopene and tomato extract to inhibit the lipid peroxidation by scavenging free radical, blocking lipid chain reaction and have a stabilizing effect supported by reduced serum CPKMB [48]. Hijazi et al. (2019) [49] reported that phenolic and flavonoid compounds of *Achillea fragrantissima* extract improve the adriamycin-induced cardiotoxicity as an antioxidant anti-inflammatory. Ashour et al. (2011) also found that bilberry (*Vaccinium*

myrtillus) extract containing anthocyanins, flavonoids, phenolic acids as antioxidant to inhibit oxidative process in doxorubicin-induced oxidative cardiotoxicity in rats. In this present study, 400 mg/kg BW of *P. emblica* L. extract in the DOX-induced rats' body showed a remarkable reduction of CK-MB and repairment in heart tissue. This findings are in line with previous study that reported the heart tissue improving by *P. emblica* L. extracts with a various concentration in doxorubicin-induced cardiotoxicity due to the antioxidant activity of *Phyllanthus emblica* L. [51]–[54].

CONCLUSION

Phytochemical screening of ethanol extract of *Phyllanthus emblica* L. contained secondary metabolites such as alkaloids, flavonoids, phenolics, triterpenoids, and tannins. Doxorubicin-induced rats' body with 400 mg/kg BW dose of *P. emblica* L. extracts showed remarkably decreasing CK-MB level. It showed the great improvements in regular arrangement and shape of myocardial muscle cells of cardiac tissue. This study showed the potential protective effect of *P. emblica* L. against doxorubicin-induced cardiotoxicity in Male Wistar Rats.

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Conflict Interests

There is no conflict of interest in this work.

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