

Cardioprotective Effect of Chloroform Extract of *Arcangelisia flava* on Doxorubicin-induced Cardiomyopathy

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ABSTRACT

Long-term use of doxorubicin as cancer chemotherapeutic agent would cause tissue toxicity, including cardiotoxicity. *Arcangelisia flava* is suggested to have cardio protective effect. This study aimed to determine the effect of chloroform extract of *A. flava* leaves on cardio histopathology of doxorubicin-treated Wistar white male rats. Wistar male rats were divided into four groups (1) control group; (2) doxorubicin 7.5 mg/kgBW intraperitoneally twice (day 1 and 6); (3) doxorubicin + chloroform extract of *A. flava* leaves 250 mg/kgBW/day orally for 11 days; (4) Chloroform extract of *A. flava* leaves 250 mg/kgBW/day orally for 11 days. At the 12th day, the rats were sacrificed; the heart organ was taken to make histopathological preparations and analyzed using HE staining. Vacuolization and necrosis are the parameters used in evaluating this effect. The phytochemical screening was also done to determine the compounds in chloroform extract of *A. flava* leaves. Based on the HE staining, chloroform extract of *A. flava* leaves decreased the cardiotoxicity caused by doxorubicin. The phytochemical screening showed that chloroform extract of *A. flava* leaves contains flavonoid, tannin, alkaloid, and triterpenoid. The cardioprotective effect of chloroform extract of *A. flava* leaves was suggested to be contributed by the flavonoid, tannin, and triterpenoid.

Keywords: chloroform extract of *Arcangelisia flava*, doxorubicin-treated rat, cardio protective, phytochemical screening.

INTRODUCTION

Cancer is caused by the abnormal and uncontrolled body tissue cells growth. WHO reports that 1 cancer patient dies every 11 minutes and new cancer case appears every 3 minutes (Brundtland, 2003). Cancer could be prevented by avoiding its triggers and improving diet. Cancer treatments mostly applied are chemotherapy, radiotherapy, surgery, and hormones therapy (Fong & Tse-Ling, 2002). Chemotherapy is a method uses chemical compounds to suppress or stop cell proliferation, and destroy cancer cells (cytotoxic). Doxorubicin is an anthracycline antibiotic that is considered to be effective as an anticancer chemotherapy agent and is widely used. But it provides several side effects including immune system depletion, hair loss, sore throat, hepatotoxicity, and irreversible cardiotoxicity or hepatotoxicity (Frias *et al.*, 2010).

The search of anticancer agent is still progressing. One potentially useful plant for anticancer therapy is *Arcangelisia flava* (Keawpradub *et al.*, 2004), and endemic plant found in Meru Betiri National Park, Jember. Based on Keawpradub *et al.* (2004), chloroform extract of *A. flava* leaves has antioxidant and cytotoxic effect on brine shrimp larvae and

MCF-7 breast cancer cells. chloroform extract of *A. flava* leaves contains berberine, palmatin, and jatrorrhizin, flavonoids, and saponins (Maryani *et al.*, 2013). The saponin in chloroform extract of *A. flava* leaves is known to provide hepatoprotective activity in rat liver induced by paracetamol with an antioxidant mechanism (Achmadi *et al.*, 2006). Flavonoids are able to suppress the formation of reactive oxygen species (ROS), and chelate metal ions (Scalbert *et al.*, 2005). Chloroform extract of *A. flava* leaves is potential as a co-chemotherapy agent to reduce the cardiotoxic effects of doxorubicin.

This study aimed to determine the effect of chloroform extract of *A. flava* leaves on the cardio histopathology on white male Wistar strain rat induced by doxorubicin.

METHODS

Materials

A. flava leaves were obtained from Meru Betiri National Park, doxorubicin was obtained from the Regional Hospital dr. Soebandi Jember, solvent n-hexane, chloroform, formalin 10%, hematoxylin eosin, xylol, and liquid paraffin. The test animals were Wistar strain male white rats, aged 8-10 weeks, weighing 140 ± 30 grams. The test animals were kept in standard conditions and fed with standard commercial pellets and ad libitum as drink.

Preparation of chloroform extract

Chloroform extract of *A. flava* leaves was made based on research by Keawpradub *et al.* (2004) with a slight modification. *A. flava* leaves were sorted and dried, then grinded into powder form. A total of 1 kg dried leaf powder was extracted successively with n-hexane and chloroform, each with 3 replications. The chloroform extract was then concentrated using a rotary evaporator and dried in an oven at 50°C. A suspension of chloroform extract of *A. flava* leaves was made in 0.5% CMC Na before use.

Animal experimental

All test animals were acclimatized for 10 days in a cage before given any treatment. Test animals were divided into 4 groups of 5 mice each. Group I, the control group, was given a 0.5% CMC Na solution (po) for 11 days. Group II was given doxorubicin (7.5 mg/kg BW) (ip) twice in 11 days (day 1 and day 6). Group III was given chloroform extract of *A. flava* leaves (po) at a dose of 250 mg/kg BW/day every day for 11 days and doxorubicin (7.5 mg/kg BW) (ip) twice in 11 days (day 1 and day 6). Group IV was given chloroform extract of *A. flava* leaves (po) at a dose of 500 mg/kg BW/day every day for 11 days and doxorubicin (7.5 mg/kg BW) (ip) twice in 11 days (day 1 and day 6) (Raksamiharja *et al.*, 2012).

Cardio histopathology

On the 12th day, the rats were sacrificed, then dissected and their hearts were taken to make histopathological microscope slide set. The heart was cleaned from the fat, washed with distilled water, dried using tissue, and fixed in 10% formalin solution to make histopathological microscope slide set using the paraffin method with the following procedure: fixation stage using formalin 10%; the dehydration stage using gradient alcohol concentrations (70, 80, 95, to 100%); the clearing stage using xylol; the impregnation stage at a temperature of 56-60°C; and the embedding stage using paraffin at 56-60°C. The samples were then stored in the freezer until it freezes.

The cutting was carried out using a microtome blade. Then, the tissue was stained using hematoxylin eosin (HE) method. The colors obtained are a blue cell nucleus and red cytoplasm. The final result were observed under a light microscope under 400 and 1,000 X magnification, and photographed. The degree of tissue damage was determined based on the Billingham scoring (1978).

Data analysis

The HE histopathological preparations was observed based on Billingham scoring system (Billingham *et al.*, 1978) as described in Table 1.

Statistical analysis was performed using Kruskal-Wallis, followed by post-hoc tests (using Mann-Whitney) for multi-group comparisons. $p < 0.05$ was considered as statistically significant.

Table 1. Billingham scoring system (Billingham *et al.*, 1978)

Score	Description
0	Normal
0,5	Not completely normal, but no evidence of anthracycline-induced damaged.
1	Myocyte cell necrosis <5% of all cells.
1,5	Myocyte cell necrosis 6-15% of all cells
2	Myocyte cell necrosis 16-25% of all cells. Myocyte cell necrosis occurs in one cluster.
2,5	Myocyte cell necrosis 26-35% of all cells. Myocyte affected by vacuolization.
3	Myocyte cell necrosis >35% of all cells.

Phytochemical screening

Identification of compounds in the chloroform extract of *A. flava* leaves was done using thin layer chromatography. A total sample of 20 mg was dissolved in 10 ml of chloroform and examined using TLC

Stationary phase: kiesel gel GF 254

Mobile phase: toluene - ethanol - NH₃ 25% (4: 3: 1).

Flavonoid Identification

Given the appearance of ammonia and citroborate vapor stains, evaluated on visual observations using UV-365 nm. The indications of the flavonoids presence in the extract are yellowish-green spots appear on visual observations, and red-orange under UV-365 nm (Robinson, 1995).

Tannin Identification

FeCl₃ stain was used to detect tannin. Black color appearance on visual observations indicates the presence of tannin (Fong & Tse-Ling, 2002).

Alkaloid Identification

Given the appearance of dragendorf stain to detect the presence of alkaloids (Wagner & Grevel, 1982). Positive results of alkaloids are shown by the presence of orange-brownish brown stains on visual observation (Pascual *et al.*, 2002).

Triterpenoid identification

Given the appearance of anisaldehyde reagent stains, then the plates are heated, and evaluated on visual observations. If a red-violet color appears, it indicates the triterpenoids presence (Wagner & Grevel, 1982).

RESULTS AND DISCUSSION

Cardio histopathology observation

The results of microscopic observations representing each treatment group are shown in Figure 1. The microscope slide sets are cardiac preparations; cross section; with HE staining which shows the difference in the number of necrotic myocardial cells between treatments. The limit of one value variation to another is not clear so only the number of necrotic myocardial cells and vacuolization are comparable.

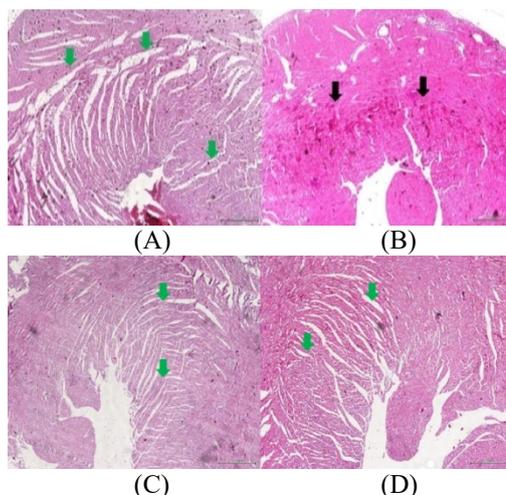


Figure 1. Cardio histopathological preparation (40X mag.) of (A) control; (B) doxorubicin (7,5 mg/kgBB); (C) chloroform extract of *A. flava* leaves 250 mg/kgBW + doxorubicin; (D) chloroform extract of *A. flava* leaves 500 mg/kgBW + doxorubicin. Normal myocardial cells with clearly demarcated cell walls (green arrows); abnormal cells with unclear cell wall boundaries (black arrows)

The cardio histopathology preparation in the doxorubicin group (400X mag.) showed a cytoplasmic image that tends to be eosinophilic (reddish) with homogeneous color features (Figure 2). Yellow arrows pointing at the coagulated or compacted cell nucleus, which is called picnotics.

The more detailed observations of rat heart muscle cells were carried out at 1.000X magnification (Figure 3). It showed that the cardiac muscle cells that were induced by doxorubicin have vacuolized (red arrows).

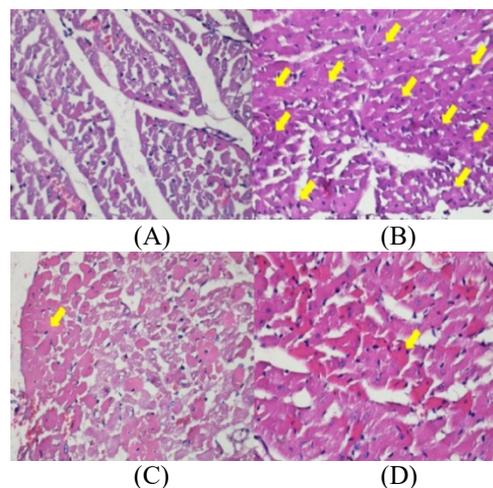


Figure 2. Cardio histopathological preparation (400X mag.) of (A) control; (B) doxorubicin (7,5 mg/kgBW); (C) chloroform extract of *A. flava* leaves 250 mg/kgBW + doxorubicin; (D) chloroform extract of *A. flava* 500 mg/kgBW + doxorubicin. Myocardial cells generally experience coagulative death (coagulative necrosis) which is characterized by a picture of the eosinophilic cytoplasm (yellow arrows) and decreases due to the myofilamen coagulation process.

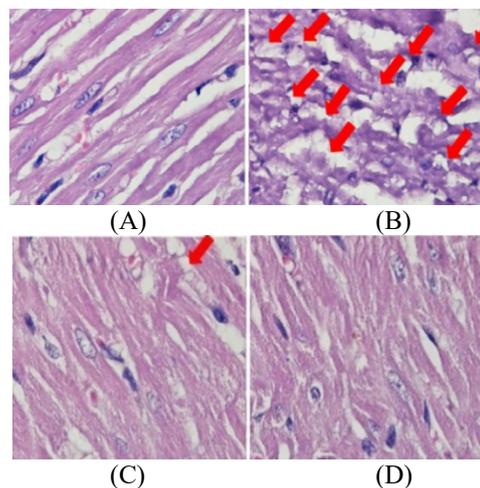


Figure 3. Cardio histopathological preparation (1,000X mag.) of (A) control; (B) doxorubicin (7,5 mg/kgBW); (C) chloroform extract of *A. flava* leaves 250 mg/kgBW + doxorubicin; (D) chloroform extract of *A. flava* 500 mg/kgBW + doxorubicin. Myocardial cells have vacuolized (red arrows)

Observations of cardiac muscle cells were analyzed in accordance with the Billingham scoring degree (1978) as seen at Figure 4. The higher the score value, the more severe the damage occurs. The group that received doxorubicin had more severe cell damage (necrosis and vacuolization) than that of the control group. Chloroform extract of *A. flava* leaves was proven to reduce damage on heart muscle cells. Due to administration of doxorubicin, the decreasing damage of heart muscle cells was in line with chloroform extract of *A. flava* leaves dose. The greater the chloroform extract of *A. flava* leaves dose, the greater the heart muscle cells repair. The chloroform extract of *A. flava* leaves dose of 500 mg/kg BW showed a histopathological profile of rat heart muscle cells with no difference from that of the control group.

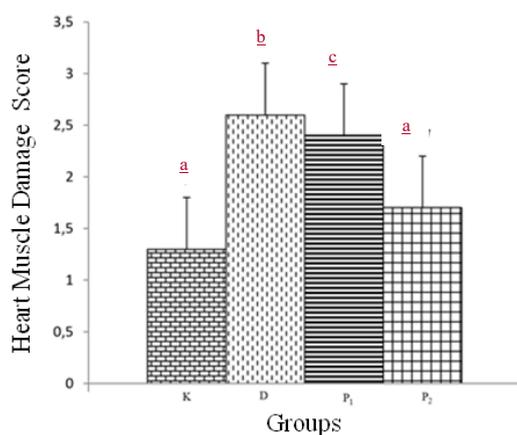


Figure 4. Mean scoring of rat heart muscle cells. (K) control; (D) doxorubicin (7.5 mg/kgBW); (P₁) chloroform extract of *A. flava* leaves 250 mg/kgBW + doxorubicin; (P₂) chloroform extract of *A. flava* leaves 500 mg/kgBW + doxorubicin. Differences in letter notation indicate significant differences ($p < 0,05$; $n = 5$)

Cancer therapy with chemotherapy agents such as doxorubicin can cause several side effects, including nausea, vomiting, myelosuppression, hair loss, and cardiotoxicity side effects that can limit the potential use of doxorubicin therapy. Cardiotoxicity is the side effect that depends on the high dose of doxorubicin. Cardiotoxicity can also cause permanent damage to the heart organ that leads to the development of deadly heart failure. One

of the cardiotoxic effects is cardiomyopathy; a decrease in myocardial function caused by changes in the histological structure of the myocardium. In the cardiomyopathy case, histologically it will show cardiac atrophy, nuclear pycnotic, myocardial edema and cytoplasmic vacuole (Pontes *et al.*, 2010).

Histopathological examination of the normal rat's heart showed a clear picture of the myocardial cell membrane structure (Fig. 1). The heart muscle cells damage caused by doxorubicin is indicated by the presence of necrosis and vacuolization. The picture of necrosis and vacuolization in the doxorubicin group showed significantly different results compared to the control group. The administration of chloroform extract of *A. flava* leaves was proven to reduce heart muscle cell damage which is in line with the large dose of chloroform extract of *A. flava* leaves. The greater the chloroform extract of *A. flava* leaves dose, the greater the heart muscle cells repair.

The chloroform extract of *A. flava* leaves group with a dose of 500 mg/kg BW did not show significantly different results from that of the control group, meaning that the dose was able to prevent or repair damaged heart muscle cells due to doxorubicin administration. Therefore, it opens up opportunities for the use of chloroform extract of *A. flava* leaves as a doxorubicin co-chemotherapy agent, primarily aimed at reducing the side effects of cardiotoxicity.

Phytochemical screening

Phytochemical screening was carried out to determine whether chloroform extract of *A. flava* leaves contains flavonoids, tannins, alkaloids, and triterpenoids by TLC method (Table 2). Phytochemical screening results showed that chloroform extract of *A. flava* leaves contains flavonoid, tannin, alkaloid, and triterpenoid compounds.

Flavonoid and tannin compounds have antioxidant activity (Scalbert *et al.*, 2005). Tannins have antioxidant effect, therefore it provides cardioprotective activity. Based on Panchal & Brown (2013), the tannin content in *Quercus petraea* extract can improve the profile of biochemical parameters in rat heart muscle cells exposed to doxorubicin. Tannin cardioprotective activity is caused by free radical scavenging activity, activation of antioxidant enzymes (Mamta & Sashi, 2012), and lipid peroxidation inhibitors inside tannins (Oliboni *et al.*, 2011).

Table 2. Phytochemical screening of chloroform extract of *A. flava* leaves

Compound	Identification		Info*
	Stain appeared	Result	
Flavonoids	Ammonia and citroborate vapor	yellowish-green on visual observation, and orange red color on UV 365 nm black color on visual observation	+
Tannin	FeCl ₃	orange-brown on visual observation	+
Alkaloids	Dragendorf	red-violet on visual observation	+
Triterpenoid	Anisaldehyde	visual observation	+

*(+) = detected; (-) = not detected

Flavonoids can suppress the formation of ROS, chelate metal ions, and regenerate antioxidant bound bonds (Scalbert *et al.*, 2005). The pharmacological effects of flavonoids that provide cardioprotective effects on rat heart muscle cells exposed to doxorubicin are antioxidant and chelating effects (van Acker *et al.*, 2001). Those flavonoids activities are associated with the inhibition of oxidative stress due to excessive production of ROS. It causes a Fenton reaction which produces hydroxyl ions (OH⁺), which is highly reactive to all of the molecules causing the formation of lipid peroxides, oxidized protein, and DNA oxidation (Takemura & Fujiwara, 2007). The activity of flavonoids as chelating iron will chelate iron Fe²⁺ so it would not form Fe which triggers the formation of the Fenton reaction (Mira *et al.*, 2002). Therefore, chloroform extract of *A. flava* leaves may be used as a doxorubicin co-chemotherapy agent because it reduces the cardiotoxic side effects of doxorubicin by reducing damage to heart muscle cells based on the histopathological profile.

Based on the results of phytochemical screening, it showed that the chloroform extract of *A. flava* leaves contains triterpenoids. According to research by Shanmugarajan *et al.*, (2008), triterpenoids from *Ficus hypida* could increase the reduction in glutathione (GSH) in rat cardiotoxicity of rat-induced cyclophosphamide chemotherapy agents. GSH is the first defense against ROS derived from endogenous sulfhydryl (SH). Triterpenoid β-

amirin compounds play a role in increasing GSH, they can provide cardioprotective effects in the rats exposed to cyclophosphamide.

The results of this study indicate that chloroform extract of *A. flava* leaves has the potential as a co-chemotherapy agent to reduce the side effects of doxorubicin cardiotoxicity as seen from the histopathological profile of the heart muscle cells of doxorubicin-induced male Wistar strain rats. Based on the research by Shanmugarajan *et al.* (2008), groups of flavonoid compounds, tannins, and triterpenoids have cardioprotective effects on chemotherapy agents-induced rats. The the presence of flavonoid, tannin, and triterpenoid on chloroform extract of *A. flava* leaves suggested to contribute on the cardioprotective effects.

Cardioprotective dose which gives a significant change is chloroform extract of *A. flava* leaves at the dose of 500 mg/kg BW. It proves that the higher the extract dose, the greater the levels of flavonoids, tannins, and triterpenoids, and will give higher cardioprotective effect. The chloroform extract of *A. flava* leaves has the potential to be developed as a co-chemotherapy agent for doxorubicin. However, whether or not this extract can increase the effectiveness of chemotherapy, further research is needed to find out the chloroform extract of *A. flava* leaves's safety and toxicity, and also a research using biochemical parameters of cardiac muscle cell damage (CK-MB and troponin).

CONCLUSION

Chloroform extract of *A. flava* leaves 250 mg/kg BW and 500 mg/kg BW can reduce the cardiotoxicity of doxorubicin in the heart of male Wistar strain rats. The reduction of cardiotoxicity is dose dependent. The dose of chloroform extract of *A. flava* leaves 500 mg/kg BW showed histopathological profiles of rat heart muscle cells which are similar with the control group.

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