This study was carried out to investigate phytochemical screening of ethylacetate extract of *Zanthoxylum acanthopodium* DC Lour. fruit (EEZ) and cardioprotective effect against doxorubicin-induced cardiomyopathy in rats. EEZ was prepared by maceration and 300 mg/kg bw as dosage of extract, then cardioprotective effects against doxorubicin (DOX) induced cardiotoxicity was evaluated. DOX was administered to rats at dose of 20 mg/bw through intraperitoneal route for two days. Cardioprotective effect was evaluated by measuring biomarkers troponin T (cTnT), CK-MB levels and histopathology of rat's heart tissue was examined. Result of phytochemical screening of extract was found to contain alkaloids, flavonoids, tannin, glycosides, and saponin. Levels of cTnT and CK-MB of DOX group differ significantly from the control group, EEZ, EEZ + DOX, vitamin E + DOX, and rutine + DOX (p <0.05). DOX raised cTnT and CK-MB levels significantly (p<0,05) and were counteracted by administration of vitamin E, rutin, and EEZ. Histopathological analysis of rat’s heart tissue resulted in myocytolysis with congestion of blood vessels, pyknosis, cytoplasmic vacuolization and fragmentation. Concomitant treatment with vitamin E, rutin, and EEZ revealed normal muscle fiber. This results suggest that EEZ has cardioprotective effect.

**Keywords**: Doxorubicin, EEZ, vitamin E, rutin, cardioprotective effect

**Introduction**

*Zanthoxylum acanthopodium* DC is an ethnic plant include *Zanthoxylum* genus, Rutaceae family. Andaliman fruit contains many compounds, those are antioxidants. The flavonoid compounds are active as a protector for myocardium cells by inhibiting the action of DOX as iron chelation, antioxidant activity, and inhibit carbonyl reductase. Alkaloids has potential as antioxidant activity. Doxorubicin (DOX) is an anthracycline class of the most effective and broad-spectrum antineoplastic widely used as anticancer on various types of cancer including breast cancer but the use of DOX is clinically irreversible cardiotoxic side effects and cause of death in cancer patients. Therefore, the use of DOX has been restricted for the purpose of minimizing the incidence of cardiotoxic, however, efficacy as an antitumor decrease. The
mechanism of DOX as the cause of cardiotoxicity that is through the formation of free radicals associated with iron and metabolites doxorubicinol\(^{12,13}\).

Myocardium is an organ that is more sensitive to free radicals produced by DOX as a source of endogenous enzymatic antioxidant in the heart, such as *superoxide dismutase* (*SOD*), *glutathione peroxidase*, catalase, dan glutathion reductase (*GSH*) resulting in irreversible damage to the myocardium cells \(^{14,15,16}\). Cardioprotective effects of any compound are indicated by measuring levels of biomarkers such as cTnT and CK-MB.

The purpose of this study was to investigate the cardioprotective effect of ethylacetate extract of *Zanthoxylum acanthopodium* DC Lour. fruit (EEZ) in female rats induced doxorubicin.

**MATERIALS AND METHODS**

**Materials**

Doxorubicin (DOX), Ketamine, *Zanthoxylum acanthopodium* DC Lour fruit was obtained from from Onan Rungu village, Samosir regency, Sumatera Utara province, Indonesia. *Zanthoxylum acanthopodium* DC Lour. was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium. CMC Na, Female Rats (*Rattus norvegicus*) 200-250 body weight.

**Preparation of EEZ**

The air-dried and powdered fruit of *Zanthoxylum acanthopodium* DC Lour. (1 kg) were repeatedly extracted by cold maceration with n-hexane (3x3 d, 7.5 L). The powder was dried in the air and extracted with ethyl acetate (3x3 d, 7.5 L) at room temperature on a shake. The filtrate was collected, and then evaporated under reduced pressure to give a viscous extract and then freeze dried to give a dried extract\(^{17}\).

**Determining Class of Chemical Compound**

Determining the class of chemical compounds carried out on simplex and EEZ\(^{18,19}\).

**Experimental Design**

The animals were divided into six groups; each group consisting of five rats: Group 1: Rats were injected with CMC Na (negative control). Group 2: Rats received EEZ (300 mg/kg) orally for nine consecutive days. Group 3: Rats in this group was treated intraperitoneally with a single dose (20 mg/kg) of DOX. Group 4: Rats received EEZ (300 mg/kg) orally started 7 days before DOX (20 mg/kg) administration and continued for the next two consecutive days. Group 5: Rats received rutin 50 mg/kg BB and DOX (20 mg/kg). Group 6: Rats received vitamin E 100 mg/kg BB and DOX (20 mg/kg). The administration of each treatment and DOX based on group 4. At the end of the experiment, the rats were anesthesied by ketamine; blood samples were collected into tubes and measured cTnT and CK-MB levels. The hearts were removed, cleaned and washed in ice-cold physiological saline and then fixed in 10% buffered formalin solution at room temperature for histopathological evaluation\(^{20,21}\). 22,23,24,25
RESULTS

The results of phytochemical screening is presented in Table 1. As can be seen in Table 1, it is shown that the result of phytochemical screening. The result determining the class of chemical compounds of Zanthoxylum achantopodium DC Lour simplex show presence of alkaloids, flavonoids, saponins, glycosides, antrakuinon glycosides and triterpenoids/steroids group compound. In EEZ show presence of alkaloids, flavonoids, saponins, glycosides group compound, and tannin.

Table 1: The result of phytochemical screening

<table>
<thead>
<tr>
<th>No</th>
<th>Screening</th>
<th>Simplex</th>
<th>EEZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Saponins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Antrakuinon glycoside</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Tannins</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Triterpenoid/steroid</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The results of treatments on cTnT and CK-MB level was presented in Table 2, Fig 1 and 2. Based on the results obtained, there was no significant difference between cTnT levels with EEZ and control group (p>0.05) but the levels of CK-MB, control group differed significantly with EEZ group (p<0.05). Levels of cTnT and CK-MB DOX group differ significantly from the control group, the group EEZ + DOX, DOX + vitamin E group and rutin + DOX group (p<0.05).

Table 2: The result of treatments on cTnT and CK-MB level

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cTnT (µg/L)</th>
<th>CK-MB (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CMC Na)</td>
<td>0.31 ± 0.05°</td>
<td>126 ± 5.29°</td>
</tr>
<tr>
<td>DOX</td>
<td>1.89 ± 0.18°</td>
<td>321 ± 7.93°</td>
</tr>
<tr>
<td>EEZ</td>
<td>0.15 ± 0.05°</td>
<td>132 ± 15.59°</td>
</tr>
<tr>
<td>Vitamin E +</td>
<td>0.23 ± 0.01°</td>
<td>135 ± 3.60°</td>
</tr>
</tbody>
</table>

| DOX       | 0.10 ± 0.11° | 122 ± 4.18° |
| EEZ + DOX | 0.26 ± 0.09° | 138 ± 8.32° |

Notes:
a: Sig (p) < 0.05 → there are significant differences with DOX group
b: Sig (p) < 0.05 → there are significant differences with control group

The results of histopathological examination of heart by HE (haematoxyllin-eosin) staining is presented in Figure 3. Based on these results, in the control group treated with CMC Na 0,5% did not cause damage to heart muscle cells (normal forms) and the boundary between the cells of the heart muscle fibers was clear and regular. In the group of DOX appeared bleeding, irregular heart muscle fibers and muscle fiber fragmentation. There were heart muscle cells that underwent pyknosis. In the group of EEZ found that the structure of the heart muscle cells was similar to the shape of normal cells in the treatment of CMC Na although there was a pyknosis. The group of EEZ which induced DOX appeared miocytolycis. In the group of rutin+DOX, appeared that the normal cardiac muscle cell structure but had a little bleeding. There were regeneration tissue. In the group of DOX induced vitamin E, it appeared that the structure of normal miofibril and there was no damage to the myocytes.

DISCUSSION

EEZ was given orally in female rats during the 7 days prior to doxorubicin-induced, and the next 2 days followed by administration of the extract 1 hour before inducing of doxorubicin. This was done with the so that antioxidant compounds of EEZ distributed in heart mitochondria. In normal condition
the source of endogenous enzymatic antioxidant in the heart, such as superoxide dismutase (SOD), glutathione peroxidase, catalase, and glutathione reductase (GSH) are lower\textsuperscript{14,15,16}. Doxorubicin was administered intramuscularly in female rats at a dose of 20 mg/kg bw\textsuperscript{26}. Based on previous studies, the dose of doxorubicin 20 mg/kg bw may lead to cardiotoxic\textsuperscript{26,27}.

There was no significant difference between cTnT and CK-MB levels between EEZ and control group (p > 0.05). cTnT levels is more specific to describe the damage on the heart than CKMB levels\textsuperscript{28}. This shows that EEZ did not affect the levels of cTnT. Levels of cTnT and CK-MB in DOX group differed significantly from the control, EEZ + DOX, Vitamin E + DOX and rutin + DOX (p <0.05). Based on these results that EEZ, vitamin E and rutin can reduce levels of cTnT and CK-MB in DOX-induced.

EEZ containing flavonoids and alkaloids, are active as protector of the cardiotoxic, inhibit DOX action. by acting as iron chelation, antioxidant activity, and inhibits carbonyl reductase\textsuperscript{3}. Flavonoids inhibit Xanthine oxidase, cyclooxygenase, microsomal succinoxidase, lipoxygenase, and NADH oxidase. Flavonoids also have an inhibitory effect on the expression of inducible nitric oxide synthase (NOS) but did not inhibit its activity\textsuperscript{29}. Nitric oxide synthases and NAD(P)H oxidase are involved in the formation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) from DOX metabolism\textsuperscript{30}.

Rutin is a flavonoid compound that acts also as a cardioprotective by means of iron chelate complex formation, antioxidant activity and inhibition of enzymes that play a role in the formation of ROS and DOX metabolism such as nitric oxide synthases, NAD(P)H oxidase and carbonyl reductases that inhibit the activity of free radicals resulting from doxorubicin. Flavonoid compounds act as well as iron chelation, which can reduce levels of iron in the mitochondria of the heart, therefore, it can protect the heart from the effects of DOX-induced cardiomyopathy\textsuperscript{31}. Flavonoids also play a role in neutralizing ROS such as hydroxyl radicals, superoxide anion radicals, hydrogen peroxide, nitric oxide radicals and lipid.

**Figure 1:** Effect of each treatments on cTnT levels
Vitamin E is a neutralizing antioxidants play a role in inhibiting lipid peroxide (RO$_2^-$). By this action, vitamin E possibly has a cardioprotective effect as well.

Based on the results of histopathological examination of heart by HE staining, a control group treated with CMC Na 0.5% did not seemed causing damage heart muscle cells (normal forms) and the boundary between the cells of the heart muscle fibers clear and regular. In the group of DOX seemed to be bleeding, irregular heart muscle fibers and muscle fiber fragmentation. There were heart muscle cells that underwent pyknosis. Tissue heart muscle cells are particularly vulnerable to free radicals. Free radicals produced from DOX reacts with unsaturated fatty acids to form lipid peroxides, a conjugate diene and malonidialdehyde. As a result, changing the structure of lipid bilayer membranes causing cell damage accompanied by cell death. ROS can affect the proteins and nucleic acids, in particular ion channels and ion transporters.$^{13}$ Oxidative stress also affects Ca$^{2+}$ homeostasis directly through the induction of mitochondrial permeability transition with changes in calcium transport in mitochondria. Changes in calcium transport can cause tissue damage, cell death and impaired contraction of the heart.$^{34}$ Due to this group of DOX-induced myocardial tissue structures demaged.
**Figure 3:** Result of heart histopathology.

- **a:** Normal myositis
- **b:** Congestion of blood vessels
- **c:** Pyknosis
- **m:** Myocytolysis
- **f:** Fragmentation
- **g:** Miofibril normal
- **h:** Karyolysis
- **x:** Tissue regeneration

In the group EEZ shown that the structure of the heart muscle cells is similar to the shape of normal cells in the treatment of CMC Na. It is clear that there is no influence of EEZ to damage heart muscle cells. While on EEZ + DOX group, it appeared that the structure of cardiac muscle cells underwent minimal bleeding, and damage to the heart muscle cells (fragmentation and miofibrilosis). This suggests that the role of free radicals can be reduced by EEZ when compared with DOX group.
In rutin + DOX, it appeared that the normal cardiac muscle cell structure and no damage but had a little bleeding. This suggests that the role of free radicals can be suppressed by the antioxidant activity of rutin.

In vitamin E + DOX, it appeared that the structure of normal heart muscle cells and not damage to the myocytes. Vitamin E is a neutralizing antioxidants play a role in inhibiting lipid peroxide (RO$_2^*$)$^{32}$ so that preventing damage cell membranes of the myocardium. Therefore, vitamin E has a cardioprotective effect which protects the heart muscle tissue.

CONCLUSION
According to the result obtained, EEZ is potential as cardioprotective by decreasing of cTnT and CK-MB levels and protecting cardiomyocyte.

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References


