Cardioprotective Effect of Ethylacetate Extract of Poguntano (Picria fel-terrae Lour.) Against Doxorubicin-Induced Cardiotoxicity in Rats

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ABSTRACT
This study was carried out to investigate phytochemical screening of ethylacetate extract of Picria fel-terrae Lour. leaves (EEP) and cardioprotective effect against doxorubicin-induced cardiomyopathy in rats. Dry EEP was prepared from dry grounded leaves by cold maceration, and extract was given at dosage of 300 mg/kg bw, then it’s cardioprotective effects against doxorubicin (DOX) induced cardiotoxicity was evaluated. DOX was administered intraperitoneally to rats at dosage of 20 mg/bw once a day for two days. Cardioprotective effect was evaluated by measuring biomarkers troponin T (cTnT), CK-MB levels. Histopathology of rat’s heart tissue was also examined. Result of phytochemical screening of extract was found to contain flavonoids, tannins, glycosides, and saponins. Levels of cTnT and CK-MB in DOX group are significantly different from that in control group, EEP, EEP + DOX, vitamin E + DOX, and rutine + DOX (p <0.05). DOX significantly raised cTnT and CK-MB levels (p<0,05) but decreased after administration of vitamin E, rutin, and EEP. Histopathological analysis of rat’s heart tissue showed to resulted in myocytolysis with congestion of blood vessels, pyknosis, cytoplasmic vacuolization and fragmentation. Concomitant treatment with vitamin E, rutin, and EEP revealed normal muscle fiber. These results suggest that EEP has cardioprotective effect.

Keywords: Doxorubicin, EEP, vitamin E, rutin, cardioprotective effect

Introduction
Doxorubicin (DOX) is an anthracycline known to be the most effective and broad-spectrum antineoplastic widely used as anticancer on various types of cancer including breast cancer¹,². However the use of DOX has clinically irreversible cardiotoxic side effects such as contraction dysfunction, congestive heart failure, therefore DOX is one of the causes of death in cancer patients³,⁴,⁵,⁶. Because of that, the use of DOX has been restricted in order to minimize the incidence of cardiotoxic, however, efficacy as an antitumor decrease²,⁷. The mechanism by which DOX may cause cardiotoxicity is that through the formation of free radicals associated with iron and metabolites doxorubicinol⁸,⁹.

Compared to the other organs such as liver and kidney, myocardium produces lower endogenous enzymatic antioxidant in the heart, such as superoxide dismutase (SOD), glutathione
peroxidase, catalase, and glutathione reductase (GSH), and hence is more sensitive to free radicals produced by DOX, resulting in irreversible damage to the myocardium cells

*Picria fel-terrae* Lour leaves contain antioxidants mainly as flavonoids. The flavonoid compounds are protective for myocardium cells due to its antioxidant activity and by inhibiting the action of DOX as iron chelation, and carbonyl reductase.

Cardioprotective effects of a compound can be evaluated by measuring cTnT and CK-MB levels as biomarkers. The purpose of this study was to investigate the cardioprotective effect of ethylacetate extract of *Picria fel-terrae* Lour. leaves (EEP) in female rats induced with doxorubicin by measuring cTnT and CK-MB levels as biomarkers and histopathological assessment.

**MATERIALS AND METHODS**

**Materials**

*Picria fel-terrae* Lour leaves was obtained from Tiga Lingga village, Dairi Regency, Sumatera Utara Province, Indonesia. *Picria fel-terrae* Lour., was identified in Research Centre for Biology, Indonesian Institute of Science, Bogor, and the voucher specimen was deposited in herbarium. Doxorubicin (DOX), Ketamine, CMC Na, Female Rats (*Rattus norvegicus*) with body weight of 200-250 g.

**Preparation of EEP**

The air-dried and powdered leaves of *Picria fel-terrae* 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 and occasionally with stirring. The filtrate was collected, and then evaporated under reduced pressure to give a viscous extract and then freeze dried to dry.

**Phytochemical Screening**

Identifying the group of chemical compounds present in simplex, and EEP was carried out.

**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 EEP (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 EEP (300 mg/kg) orally started for 7 days before DOX (20 mg/kg) administration and continued on the next two consecutive days. Group 5: Rats received rutin 50 mg/kg bw and DOX (20 mg/kg). Group 6: Rats received vitamin E 100 mg/kg bw and DOX (20 mg/kg). The administration of each treatment and DOX similar with that on group 4. At the end of the experiment, the rats were anesthetized by ketamine and blood samples were collected in 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.
RESULTS AND DISCUSSION

The results of phytochemical screening in simplex and EEP is presented in Table 1. As can be seen in Table 1, it is shown that the classification of chemical compounds contained in Picria felterrae Lour simplex consisted of flavonoids, saponins, glycosides, antrakuinon, glycosides and triterpenoids/steroids group. In EEP shown to contain flavonoids, saponins and glycosides, and tannin.

Table 1: The result of phytochemical screening

<table>
<thead>
<tr>
<th>No</th>
<th>Screening</th>
<th>Simplex</th>
<th>EEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoids</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>Triterpenoids/steroids</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The effects 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 affected by EEP and in control group (p> 0.05), but the levels of CK-MB in control group was significantly different from that with EEP group (p<0.05).

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

<table>
<thead>
<tr>
<th>Treatments</th>
<th>cTnT (µg/L)</th>
<th>CK-MB (UL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (CMC Na)</td>
<td>0.31 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>126 ± 5.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DOX</td>
<td>1.89 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>321 ± 7.93&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EEP</td>
<td>0.38 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>200 ± 7.37&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E + DOX</td>
<td>0.23 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>135 ± 3.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rutin + DOX</td>
<td>0.10 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>122 ± 4.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EEP + DOX</td>
<td>1.45 ± 0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>230 ± 19.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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. The control group treated with CMC Na 0.5% did not cause damage on 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 EEP group was found that the structure of the heart muscle cells was similar to that of normal cells in the treatment of CMC Na. In the EEP group in combination with DOX appeared a little bleeding, fragmentation, and miocytolysis. In the group of rutin + DOX, did not affect the normal cardiac muscle cell structure and no damage but there was a little bleeding. There is some tissue regeneration. In the group of DOX and vitamin E, it appeared that the structure of heart muscle cells was normal and there was no damage to the myocytes.
**Figure 1:** Effect of each treatment on cTnT

![Bar graph showing the level of cTnT with different treatments.]

**Figure 2:** Effect of each treatment on CK-MB

![Bar graph showing the level of CK-MB with different treatments.]

Legend:
- Control
- Dox
- EEP
- EEP + Dox
- Vit E + DOX
- Rutin + DOX
EEP was given orally in female rats during the 7 days prior to doxorubicin induction, and the next 2 days followed by administration of the extract 1 hour before doxorubicin induction. This was done in order that antioxidant compounds of EEP distributed in the heart mitochondria. In normal condition the source of endogenous enzymatic antioxidant in the heart, such as superoxide dismutase (SOD), glutathione peroxisdase, catalase, and glutathion reductase (GSH) are lower\(^\text{10,11,12}\). Doxorubicin was administered intramuscularly in female rats at a dose of 20 mg/kg bw\(^\text{23}\), because the dose of doxorubicin of 20 mg/kg bw may lead to cardiotoxic\(^\text{25,26}\).

There was no significant difference between cTnT levels between EEP and control group (p > 0.05) but the levels of CK-MB control group significantly lower than EEP group (p < 0.05). CTnT levels is more specific to describe the damage on heart than CKMB levels\(^\text{25}\). This shows that EEP did not affect the levels of cTnT. Levels of cTnT and CK-MB in DOX group different significantly from the control, EEP + DOX, Vitamin E + DOX and rutin + DOX (p < 0.05). This is to show that EEP, vitamin E and rutin can reduce levels of cTnT and CK-MB in DOX-induced.

Flavonoids present in EEP are protecting heart from cardiotoxic induced by DOX by inhibition iron chelation, active as antioxidant, and stimulates carbonyl reductase\(^\text{16}\). Flavonoids inhibit Xanthine oxidase, cycloxyegenase, 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\(^\text{28}\). 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\(^\text{7,29}\).

Rutin is a flavonoid that is 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, stimulates carbonyl reductases that inhibit the activity of free radicals resulting from doxorubicin. Flavonoids act as iron chelation, which can reduce levels of iron in the heart mitochondria, therefore, protecting the heart from the effects of cardiomyopathy induced or caused by DOX\(^\text{20}\). Flavonoids also play a role in neutralizing ROS such as hydroxyl radicals, superoxide anion radicals, hydrogen peroxide, nitric oxide radicals and lipid peroxide. Vitamin E is a neutralizing antioxidant play a role in inhibiting lipid peroxide (RO$_2$\(^\cdot\))\(^\text{31}\). 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 seem to cause damage on heart muscle cells and the boundary between the cells of the heart muscle fibers was clear and regular. In the group of DOX seemed to be bleeding, irregular heart muscle fibers and muscle fiber fragmentation. Heart muscle cells underwent pyknosis. Tissue heart muscle cells are particularly
vulnerable to free radicals. Free radicals produced from DOX reacted with unsaturated fatty acids to form lipid peroxides, a conjugate diene and malonil dialdehyde. As a result, the structure of lipid bilayer membranes changed causing cell damage accompanied by cell death. ROS can affect the proteins and nucleic acids, in particular ion channels and ion transporters\textsuperscript{32}. Oxidative stress also affects Ca\textsuperscript{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\textsuperscript{33}.

\textbf{Figure 3: Result of heart histopathology}

\begin{itemize}
\item CMC 0.5\% (control)
\item DOX
\item EEP
\item EEP + DOX
\item Vit E + DOX
\item Rutin + DOX
\end{itemize}
Notes:
a: Normal myosit
b: congestion of blood vessels
c: pyknosis
m: myocytolysis
f: fragmentation
g: miofibril normal
h: Karyolysis
x: tissue regeneration

In the group EEP, it was shown that the structure of the heart muscle cells is similar with the shape of normal cells in the treatment of CMC Na. It is clear that EEP did not cause damage on heart muscle cells. While on EEP + DOX group, it appeared that the structure of cardiac muscle cells underwent minimal bleeding, and damage to the heart muscle cells (fragmentation and myocytolysis). This suggests that the role of free radicals can be reduced by EEP when compared with DOX group.

In rutin + DOX, it appeared that there was a little bleeding but miofibril was normal and regeneration tissue emerged. There was some tissue regeneration. 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 heart muscle cells was normal and not any damage to the myocytes. Vitamin E is a neutralizing antioxidants play a role in inhibiting lipid peroxide (RO₂⁻)³¹ so that preventing damage cell membranes of the myocardium. Therefore, vitamin E has a cardioprotective effect on heart muscle tissue.

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

ACKNOWLEDGEMENTS
We gratefully thank to DP2M DIKTI (Directorate of Higher Education) Ministry of Research Technology and High Education, Indonesia through “Hibah Pascasarjana” Research Grant 2015 for financial support in the study.

References


