Correlation Between Antioxidant and Cytotoxic Activity of Parijoto (Medinilla speciosa Blume) Fractions in 4T1 Cell Line

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Abstract

Parijoto (Medinilla speciosa Blume) is one of Indonesian plant used for traditional medicine. Previous studies have demonstrated antimicrobial and cytotoxic effects of Parijoto on T47D cells. Therefore, we intended to know the antioxidant and cytotoxic activity of these fractions in 4T1 cell line (a Mus musculus mammary carcinoma). This cancer causes the greatest number of cancer-related deaths. This study also investigated the correlation between antioxidant activity and cytotoxicity of Parijoto fractions. Discovering the type of correlation between antioxidant and anticancer activity of botanical extracts could relieve in screening for cytotoxic agent from natural products. The antioxidant and cytotoxic activity investigated using the Diphenylpicrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay methods. The result showed that ethyl acetate fraction is the higher antioxidant activity (IC$_{50}$: 1.77 µg/mL) and the higher cytotoxicity (IC$_{50}$: 133.57 µg/mL). There was a strong positive correlation (correlation coefficient=0.957) between antioxidant and cytotoxic activity in 4T1 cell line, but the correlation was not significant ($p=0.188$).

Keywords: Parijoto (Medinilla speciosa Blume), antioxidant, cytotoxic, 4T1 cell line.

INTRODUCTION

The most frequent cancer among women is breast cancer. This cancer causes the greatest number of cancer-related deaths (WHO, 2018). 4T1 cell is Mus musculus mammary carcinoma. Many researchers were interested to this cancer cell line because it mimics the stage IV breast cancer in humans (Nordin, et al., 2017). This cell has the capability to metastases to several organs (Tao, et al., 2008).

During this time, cancer treatments in Indonesia are mostly done by surgery, radiation, and chemotherapy administration (Badan Penelitian dan Pengembangan Kesehatan, 2018). Patients with cancer have various reasons for wanting to try alternative therapies, including herbal medicine. Most alternative therapies have not been through such testing and there is no scientific evidence that they were worked. The development of cancer treatment is continuing to get relevant and safe therapy through herbal medicine as cytotoxic agents.

Parijoto (Medinilla speciosa Blume) is one of the Indonesian plants that grow at an altitude of minimum 600 masl (Widjanarko, 2008). In Indonesia, Parijoto grows in some areas like Muria

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Mountain and Merbabu Mountain (Widjanarko, 2008; Anggana, 2011). The utilization of Parijoto by local people is very diverse. The most known myth in Mount Muria said that if a pregnant woman consumes Parijoto, the babies will become good looking and good in the manner (Mumpuni, et al., 2015). The community also used this plant for treating diseases, such as mouth sores, diarrhea, antiinflammation, antibacterial, anticancer, on mixed or single composition (Hanum, et al., 2017). Both methanolic extract and ethyl acetate fraction of Medinilla speciose Blume contain some secondary metabolites in the form of flavonoids, saponins, tannins, glycosides. Only terpenoid found in n-hexane fraction (Niswah, 2014). Some in vitro studies found the activity of this fruit extract. Antibacterial activity for S.aureus and E.coli are showed in the inhibitory concentration of 12.5 mg/mL–200 mg/mL (Niswah, 2014). Furthermore, the ethanolic extract of Parijoto fruit has cytotoxic activity in T47D with IC_{50} 614.50 µg/mL (Tussanti, 2014). Another research conducted that the methanolic extract of Parijoto has a synergistic effect with cisplatin against HeLa cell line (Artanti, et al., 2020). We would like to find out more about the cytotoxic activity of parijoto, specifically in 4T1 cells. The 4T1 mammary carcinoma is highly tumorigenic, invasive, and metastasise from the primary tumor in the mammary gland to multiple distant sites, with lungs and liver as the principal target organs (DuPré, 2007).

Chronically increased reactive oxygen species (ROS) cause severe cellular damage and lead to carcinogenesis by modulating cell signaling in biological processes. The increase in ROS is usually accompanied by activation of the oncogene that is the first step in the transformation of malignancy (Wang and Yi, 2008). The ability of metastatic in many types of tumor also related to the level of ROS in cells. Certain metastatic stages were increased by administration of exogenous ROS (Wang and Yi, 2008). To avoid endogenous ROS overproduction, cells have a defense mechanism to eliminate ROS using exogenous and endogenous antioxidant molecules (Kim, et al., 2019). Ishikawa (2008) showed that pretreatment of the highly metastatic tumor cells with ROS scavengers suppressed their metastatic potential in mice.

Many studies have found that there are several plants that have ROS scavenger activity (antioxidant activity), which is associated with anti-proliferative activity (cytotoxic activity) on cancer cells, so they can be used as therapeutic and preventive agents (Sammar, 2019). Because of the close relationship, this study will determine the antioxidant and cytotoxic activity of the Medinilla speciosa fractions. Antioxidant and cytotoxic activity were calculated with the IC_{50} value, then the correlation between the two values will be analyzed. Discovering the type of correlation between antioxidant and anticancer activity of botanical extracts could relieve in screening for cytotoxic agent from natural products.

MATERIAL AND METHODS

Preparation of Parijoto Fraction

Medinilla speciosa Blume ripe fruits were taken from Muria Mountain Area, Colo Village, Kudus Regency, Central Java, Indonesia and identified of the plant has been done by Biology Laboratory, Faculty of Sains and Mathematics, Diponegoro University. Medinilla speciosa ripe fruits were macerated with ethanol 70% (Brataco, Jakarta, Indonesia) for 3 days. The extract was evaporated in 40°C with rotary evaporator. Purification was performed by partitioning using n-hexane, ethyl acetate, and methanol (analytical grade) (Merek, New York, USA.). All fractions were evaporated in 40°C by rotary evaporator.

Antioxidant Activity

All of the fractions were diluted in methanol. An aliquot of fraction was diluted with 0.8% Diphenylpicrylhydrazyl (DPPH) reagent on methanol (0.02 mL) and incubated for 15-30 minutes...
in dark condition. Absorbance was measured immediately by UV spectrophotometer at 520 nm. The control was absorbance of DPPH on methanol (A0), and the positive control was absorbance of Vitamin C (Himedia) diluted in DPPH on methanol (A1). The % inhibition was calculated by:

\[
\% \text{ inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100\%
\]

The IC\textsubscript{50} value was obtained by interpolating of % inhibition and concentration with the linear regression.

**Cytotoxic Activity**

Cell line (1×10\textsuperscript{5} cell/well) were cultured to 96 well plates in medium and incubated for 24 h. The samples were made in 6 series of concentration (31.25 µg/mL–1000 µg/mL). The range of Doxorubicin (Dankos, Jakarta, Indonesia) concentration was 12.5 µg/mL–200 µg/mL. Samples and positive control were added into the plates, then incubated for 24 h. A 100 µL MTT (Sigma Aldrich, St. Louis, USA) 5 mg/mL in PBS was added to each well, then incubated for 4 h until formazon formed. The reaction was stopped by adding 100 µL of 10% SDS in each well and incubated at 24 h in protected light conditions. The absorbance at 545 nm was measured using ELISA reader. Absorbance data then formulated into % inhibition using this equation:

\[
\frac{(\text{control absorbance}-\text{medium absorbance}) - (\text{sample absorbance}-\text{medium absorbance})}{(\text{control absorbance}-\text{medium absorbance})} \times 100\%
\]

Concentrations inhibiting 50% (IC\textsubscript{50}) of the cell were determined by probit analysis.

**Correlation**

Correlation between antioxidant and cytotoxic activity was analyzed by using the Pearson Correlation Coefficient.

**Ethics Approval**

Ethical clearance was obtained from Research Ethics Commission of Medical Faculty Diponegoro University with the ethical clearance approval number: 37/KEPK/FK UNDIP/VII/2019.

**RESULT**

**Antioxidant Activity**

Antioxidant activity can be shown from the ability to extract or fractions to inhibit 50% free radicals such as DPPH. Our results showed that the fraction of ethyl acetate given the highest potency as an antioxidant (1.77 µg/mL) followed by n-hexane and methanol (Table 1).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Methanol</th>
<th>Ethyl acetate</th>
<th>n-Hexane</th>
<th>Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50} (µg/mL)</td>
<td>11.27</td>
<td>1.77</td>
<td>6.01</td>
<td>10.33</td>
</tr>
</tbody>
</table>

**Cytotoxic Activity**

Cell were treated with several fractions in concentration series (31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL). Results of % inhibition was average of three independent experiments. The effect of the Parijoto fractions against 4T1 cell line was shown in Figure 1.

Cytotoxic activity of Parijoto fractions was showed in Table 2. The lower value of IC\textsubscript{50} indicated the higher potency of cytotoxic agents. The ethyl acetate fraction gave the highest potency (133.57 µg/mL) followed by n-hexane and methanol fraction.

**Correlation Between Antioxidant and Cytotoxic Activity**

The magnitude of the relationship between variables was expressed in correlation coefficient (Pearson correlation). The correlation analysis between the IC\textsubscript{50} value of antioxidant and cytotoxic showed a Pearson correlation.

**Table 2. Cytotoxic activity of Parijoto fractions.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Methanol</th>
<th>Ethyl acetate</th>
<th>n-Hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC\textsubscript{50} (µg/mL)</td>
<td>563.81</td>
<td>133.57</td>
<td>170.82</td>
</tr>
</tbody>
</table>
Figure 1. Effect of Parijoto fractions in 4T1 cell line. Cell line (1×10⁵ cell/well) were cultured to 96 well plates in medium and incubated for 24 h. The samples were made in 6 series of concentration (31.25 µg/mL–1000 µg/mL). Cells inhibition were calculated from the conversion of absorbance values obtained in 3 experiments by MTT assay. Methanol, ethyl acetate, and n-hexane were fractions of the ethanol extract from Parijoto fruit.

coefficient value of -0.957. Based on the results obtained, the significance value of the correlation between the two variables was 0.118.

DISCUSSION

Several studies mentioned that various kinds of Parijoto fractions contain phenolic and flavonoid compounds. These compounds have the ability to deactivate free radicals by donating a hydrogen atom to free radicals. This is what is known as how antioxidants work. Linear relationships between the flavonoid and the total phenolic content of some natural sources and antioxidant activity were observed (Aryal, et al., 2019).

In this study, the aim was to investigate the correlation between the antioxidant and cytotoxic activity of Parijoto in the 4T1 cell line in vitro. The results showed that all fractions of the Parijoto extract could inhibit the proliferation of 4T1 cells in several concentrations. The IC₅₀ value indicated that all fractions were moderate cytotoxic (100 µg/mL<IC₅₀<1000 µg/mL) (Prayong, et al., 2008). The Parijoto antioxidant activity was analyzed by the DPPH IC₅₀ value. The highest antioxidant and cytotoxic activity found in ethyl acetate fractions. The Pearson Correlation coefficient showed that antioxidant and cytotoxic activity of Parijoto fractions had a strong positive relationship (correlation coefficient=0.957), but that was not significant (p=0.188). Antioxidant agents generate cytotoxic activity in several ways, by enhance ROS scavenging enzymes, target NADPH oxidase, and manipulate nitroxide compounds (Wang and Ying, 2008). This insignificant correlation result of this study may be due to cytotoxic activity are not only derived from antioxidant levels, but could also be associated with the inhibitory effects via other signaling pathways. Another reason is phenolic compounds are known to have a contradictory behavior characterized by anti- and protumoral activity, depends to their chemical structure (number of OH ring substituents, saturated carbon chain lengths, and carboxylation groups) (Gomes, et al., 2003).

ROS play a role in some pathogenesis of diseases, such as diabetes, cardiovascular, and cancer. In carcinogenesis, DNA damage and changes in the formation of DNA linkages have been observed (Bennet, et al., 2012). The antioxidant can
attack and neutralize ROS and RONS (Ruberte, et al., 2020). Exogenous antioxidants have several actions to reduce ROS, there are as chain initiation protection, transition metal catalyst ion chelating, peroxidase decomposition, protection from hydrogen abstraction and radical scavenging (Valko, et al., 2006). Parijoto fractions can be developed to be prevention agent because they inhibited the ROS in low dose (1.77 µg/mL, 6.07 µg/mL and 11.27 µg/mL) and had moderate cytotoxic capabilities. Phytochemical compounds such as flavonoids and resveratrol followed by well-balanced diet rich in fruits and vegetables containing antioxidants, resulted in lower incidence of some cancer by in vitro and small clinical trials (Bennet, et al., 2012).

Previous trials have shown some benefits of antioxidants, particularly in the use of combinations of these components with chemotherapy in the treatment of cancer. Concurrent use of antioxidants and chemotherapy minimizes therapy-related side effects in several randomized control trials (Singh, 2017). The use of antioxidants in cancer patients may protect tumor cells as well as healthy cells from oxidative damage which usually caused by chemotherapeutic agents (Singh, 2017). In addition, another study demonstrated that the protection of normal tissues did not decrease tumor control (Simone, et al., 2007a, Simone, et al., 2007b).

A study has shown that methanol and ethyl acetate fraction of Parijoto have several phytochemical compounds, such as flavonoids, saponin, tanin, and glicoside. While n-hexane fraction has terpenoid (Niswah, 2014). Ethyl acetate diluted less polar flavonoids (isoflavones, flavonones, methylated flavones, and flavonols) while glycoside flavonoids can be extracted by methanol (Ferreira and Pinho, 2012). Our result showed ethyl acetate gived the higher cytotoxic activity than others might be caused by its less polar flavonoids compound. The types of flavonoid contained in Parijoto are not yet known. However, isoflavones, being one of the less polar flavonoids, have been shown the effects on cancer cells. Genistein (isoflavone from soy) inhibited growth, development, and metastasis of cancer in in vivo trial (Sahin, et al., 2019). The isoflavones was blocked tumorogenesis by some mechanism such as, inhibited enzyme for metastasis, DNA replication and signal transduction; inhibited angiogenesis growth factor such as VGEF; and activated the immune system. However, based on meta analysis study, consumption of soy was correlated with low incidence and mortality of breast cancer (Ziaei and Halaby, 2017). Therefore, our ethyl acetate fractions can be developed for rich flavonoid dietary supplement to preventing cancer agents.

CONCLUSION

This study showed a positive correlation between the antioxidant and cytotoxic activity of Parijoto fraction (correlation coefficient=0.957), but the correlation was not significant (p=0.188). Among the three fractions analyzed, ethyl acetate fraction gave the highest activity in antioxidant and cytotoxicity.

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