In Vitro Antiplasmodial Activity and Cytotoxicity of Active Subfractions of Harmsiopanax aculeatus Leaves

Rachel Turalely¹,², Mahardika Agus Wijayanti³, Triana Hertiani⁴, Mustofa⁵*

1. Biotechnology Study Program, Graduate School, Universitas Gadjah Mada, Yogyakarta, 55281 Indonesia.
2. Chemistry Education Study Program, Faculty of Teacher Training and Education Science, Pattimura University, Ambon, Maluku, 97322, Indonesia.
3. Department of Parasitology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, 55281 Indonesia.
4. Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, 55281 Indonesia.
5. Department of Pharmacology and Therapy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada Yogyakarta, 55281 Indonesia.

Info Article

Submitted: 05-12-2019
Revised: 13-01-2020
Accepted: 17-02-2020

*Corresponding author
Mustofa
Email: mustofaflk@ugm.ac.id

ABSTRACT

Harmsiopanax aculeatus leaves, a medicinal plant with locally named kapur, have been used traditionally to treat malaria in Maluku, Indonesia. However, the scientific information of this plant is still limited. In our previous study, the methanol extract of this plant leaves have been proven to possess in vitro antiplasmodial activity. This study was conducted to evaluate in vitro antiplasmodial activity and cytotoxicity of subfractions of the plant leaves. Fractionation was performed using a column chromatography with Sephadex LH-20 as the stationary phase and methanol as the mobile phase. The subfractions obtained were then tested for in vitro antiplasmodial activity on a chloroquine-resistant FCR3 strain of Plasmodium falciparum using a visual method. Cytotoxicity was evaluated by using MTT assay. The in vitro antiplasmodial activity and cytotoxicity were expressed as IC₅₀, calculated using probit analysis with SPSS 16 for windows. The results showed that the four subfractions tested have a high antiplasmodial activity with IC₅₀ values of 0.09; 0.18; 0.01; and 0.77 µg.mL⁻¹, respectively. In addition, these subfractions had IC₅₀ values of >400 µg.mL⁻¹ against Vero cells indicating that they were non-toxic. In conclusion, the subfractions of H. aculeatus leaves are very active and selective against P. falciparum. Further study will be conducted to isolate the active compounds.

Keywords: H. aculeatus, antiplasmodial activity, cytotoxicity, malaria, subfractions

INTRODUCTION

Although the number of malaria cases declined by 20% in the last decade, malaria is still one of the major public health problem worldwide, especially in tropical countries including Indonesia. In 2017, an estimated 219 million new malaria cases occurred with 435,000 deaths from malaria globally. Most malaria cases were in the African Region (92%), followed by the Southeast Asian Region (5%) and the Eastern Mediterranean Region (2%) (WHO, 2018).

Resistance to the first-line antimalarial drugs especially chloroquine is one of the major problems in malaria eradication. Currently, the World Health Organization (WHO) recommended Artemisinin-based Combination Therapies (ACTs) as first- and second-line treatment for uncomplicated or chloroquine-resistant Plasmodium (WHO, 2010). However, after several years of use, resistance to artemisinin was first reported in Cambodia in 2009 and then emerged Laos, Myanmar, Thailand and Vietnam (Fairhurst & Dondorp, 2016; Fairhurst, 2016; Wells et al., 2015).

The resistance to antimalarial drugs has encouraged the academia and the pharmaceutical industry to discover and develop new antimalarial drugs. Some strategies have been implemented through chemotype screening or identification of
synthetic target molecules in the laboratory and computationally, as well as through screening of natural resources (Wells et al., 2015).

Many medicinal plants traditionally used to treat malaria from various regions were evaluated for their potential antimalarial activity. *Harmsiopanax aculeatus* leaves, locally named *kapur*, have been used to treat malaria in Maluku, Indonesia. Previous studies were reported that methanol extract of *H. aculeatus* leaves has *in vitro* and *in vivo* antimalarial activity and it is not toxic in Vero cells line (Turalely et al., 2018; Turalely et al., 2011). Furthermore, among 12 fractions obtained from the methanol extract using chloroform-ethyl acetate (8:2), the fraction FG7 showed the most active fraction. In this study, we reported antimalarial activity and cytotoxicity of subfractions of the active fraction FG7.

**MATERIAL AND METHODS**

**Materials**

The samples of plant leaf were collected from Amahai Village, Amahai District, Central Maluku Regency, Maluku, Indonesia and determined in the Taxonomy Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia as *Harmsiopanax aculeatus* (Blume) Warb. Ex Boel (Araliaceae, Voucher number 1 HaA). The primary materials used for fractionation were Sephadex LH-20 (Sigma), dissolved fraction in chloroform-ethyl acetate (8:2), chloroform, methanol, and ethyl acetate (E-Merck), and thin-layer chromatography (TLC) plates. The primary materials for the *in vitro* antimalarial activity test were RPMI, red blood cells, *Plasmodium falciparum* strain FCR3, DMSO, human serum, and Giemsa. The primary materials for the cytotoxicity activity test were Vero cells, M199 media, DMSO, and MTT. The flavonoid test in the most active subfraction was carried out using a FeCl₃ spray reagent.

**Fractionation of dissolved fractions in chloroform-ethyl acetate (8:2)**

The dissolved fraction in chloroform-ethyl acetate (8:2) was fractionated using column chromatography with LH-20 as the stationary phase (Figure 1). The mobile phase used was 100% methanol. Thirty g of Sephadex LH-20 was soaked in methanol for 24h. Furthermore, columns measuring 1.5cm in diameter and 50cm in length, were packed using the Sephadex which had soaked up to ¼ of the column. Subsequently, the test material was dissolved using methanol and loaded on to the column. Elution was carried out using 100% methanol. Each fraction was collected at a flow rate of 2mL.min⁻¹. The collected fraction was monitored by TLC. Fractions with the same TLC profile were combined. Each fraction obtained was then tested for *in vitro* antimalarial activity and cytotoxicity.

**In vitro antimalarial activity assay**

*In vitro* antimalarial activity of each subfraction was tested against the chloroquine-resistant *P. falciparum* strain (FCR3) obtained from continuous cultured using a visual method. The Plasmodium was cultured using candle jar method according to Trager and Jensen (1976) after modification. One hundred μl of the Plasmodium culture in ring stage, after synchronized with sorbitol 5%, in a final 2% haematocrit and 0.5% parasitemia, was added into the wells of 96-well microtitre plate. Eight varies concentrations different of subfractions solution ranging from 0.005 to 60 μg.mL⁻¹, depend on each fraction, were prepared using culture medium. One hundred μl of the subfraction solution was then added in the wells in triplicate. The microtitre plate were placed in a candle jar and incubated at 37°C for 72h in a CO₂ incubator. The wells containing culture medium without subfraction were used as negative control. Followed after incubation, a thin blood smear of...
each well was prepared and then Giemsa staining was conducted. Parasitemia of each the Giemsa stained thin blood smears was observed microscopically to calculate the Plasmodium growth. Inhibitory concentration 50% (IC₅₀) or concentration that inhibit 50% Plasmodium growth, was determined using probit analysis with SPSS 16 for windows and used to express the antiplasmodial activity.

**Cytotoxicity activity assay**

The cytotoxicity of the subfractions on Vero cells line (M199) was tested using a MTT method assay method. Six varies concentrations different of subfractions solution ranging from 15.625 to 1000µg.mL⁻¹ were prepared using culture medium. One hundred µL of the cells culture and 100µL of the subfraction solutions were added into the wells of 96-well microtitre plate. The microtitre plate were incubated at 37°C for 24h in a CO₂ incubator. Each subfraction was tested in triplicate in three independent experimental. The wells containing medium culture without subfraction was used as negative control. Followed after incubation, cells medium was removed from the wells and 25µL of the MTT solution (2mg/mL in PBS) was added to each well. The microtitre plate was incubated at 37°C for 1.5h and 125µL of DMSO was added to each well to dissolve the purple formazan crystals. The absorbance of each well was measured using an ELISA reader at 595nm. The cytotoxicity was expressed as IC₅₀ calculated using probit analysis with SPSS 16 for windows.

**Phytochemical identification of the most active subfractions**

Identification of flavonoid compounds in the most active subfractions was carried out using FeCl₃ spray reagents.

**RESULT AND DISCUSSION**

Four subfractions were obtained from fractionation of 0.026g active fraction of

---

Rachel Turaley

Table I. Subfractions of *H. aculeatus* chloroform-ethyl acetate fraction and their TLC profiles

<table>
<thead>
<tr>
<th>No.</th>
<th>Subfraction</th>
<th>Form</th>
<th>% w/w</th>
<th>Rf</th>
<th>Visible light</th>
<th>UV (λ 254 nm)</th>
<th>UV (λ 366 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SF.1</td>
<td>Greenish white powder</td>
<td>0.39</td>
<td>0.86</td>
<td>-</td>
<td>Green fluorescent</td>
<td>Blue fluorescent</td>
</tr>
<tr>
<td>2</td>
<td>SF.2</td>
<td>White powder</td>
<td>38.67</td>
<td>0.88</td>
<td>-</td>
<td>Green fluorescent</td>
<td>Blue fluorescent</td>
</tr>
<tr>
<td>3</td>
<td>SF.3</td>
<td>Yellowish white powder</td>
<td>37.50</td>
<td>0.82</td>
<td>-</td>
<td>Green fluorescent</td>
<td>Blue fluorescent</td>
</tr>
<tr>
<td>4</td>
<td>SF.4</td>
<td>Greenish powder</td>
<td>23.44</td>
<td>0.79</td>
<td>-</td>
<td>Green fluorescent</td>
<td>Blue fluorescent</td>
</tr>
</tbody>
</table>

Table II. Plasmodium growth inhibition and antiplasmodial activity of subfractions of *H. aculeatus*

<table>
<thead>
<tr>
<th>Subfraction 1 (SF.1)</th>
<th>Subfraction 2 (SF.2)</th>
<th>Subfraction 3 (SF.3)</th>
<th>Subfraction 4 (SF.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (µg/mL)</td>
<td>Inhibition (%)</td>
<td>Conc. (µg/mL)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>60</td>
<td>94.89±2.87</td>
<td>100</td>
<td>78.46±15.68</td>
</tr>
<tr>
<td>50</td>
<td>94.54±3.98</td>
<td>50</td>
<td>68.97±9.09</td>
</tr>
<tr>
<td>25</td>
<td>91.57±3.54</td>
<td>10</td>
<td>61.25±7.38</td>
</tr>
<tr>
<td>20</td>
<td>86.36±12.33</td>
<td>5</td>
<td>58.87±13.97</td>
</tr>
<tr>
<td>10</td>
<td>73.01±4.83</td>
<td>1</td>
<td>55.23±5.85</td>
</tr>
<tr>
<td>5</td>
<td>62.89±2.76</td>
<td>0.5</td>
<td>52.89±8.18</td>
</tr>
<tr>
<td>1</td>
<td>57.61±8.40</td>
<td>0.1</td>
<td>46.85±3.57</td>
</tr>
<tr>
<td>0.5</td>
<td>53.53±12.26</td>
<td>0.05</td>
<td>44.67±9.11</td>
</tr>
<tr>
<td>0.1</td>
<td>49.88±4.56</td>
<td>0.01</td>
<td>41.46±6.96</td>
</tr>
<tr>
<td>0.05</td>
<td>42.68±13.86</td>
<td>0.005</td>
<td>39.15±10.71</td>
</tr>
</tbody>
</table>

IC₅₀ (µg.mL⁻¹) 0.22 0.57 0.01 0.04
chloroform-ethyl acetate. The form and TLC profile of the subfractions (Table I). The inhibition of Plasmodium growth and in vitro antimalarial activity (IC50) of the subfractions of H. aculeatus leaves (Table II), whereas their cytotoxicity and index selectivity (IS) (Table III).

Table III. Cytotoxicity and selectivity index of subfractions of H. aculeatus

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>IC50 on Vero cells (μg/mL)</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF7.1</td>
<td>&gt;700</td>
<td>&gt;7692.31</td>
</tr>
<tr>
<td>SF7.2</td>
<td>408.80</td>
<td>2271.11</td>
</tr>
<tr>
<td>SF7.3</td>
<td>&gt;1000</td>
<td>&gt;125,000</td>
</tr>
<tr>
<td>SF7.4</td>
<td>1022.44</td>
<td>131.93</td>
</tr>
</tbody>
</table>

Antiplasmodial activity of natural products or synthetic compounds can be categorized into high if the IC50 value <5 μg/mL⁻¹; promising if the IC50 value between 5-15 μg/mL⁻¹, moderate if the IC50 value between 15-50 μg/mL⁻¹ and, not active if the IC50 value >50 μg/mL⁻¹ (Jonville et al., 2008). Based on this criteria, all of the subfractions tested showed high antimalarial activity with the IC50 value <5 μg/mL⁻¹ (Table 2). The highest antimalarial activity was obtained from the SF.3 with IC50 value of 0.01 μg/mL⁻¹. Furthermore, all of the subfractions had IC50 value lower than its parent extract or fraction (methanol extract or chloroform-ethyl acetate fraction) that reported in the previous study (Turalely et al., 2018). It is indicated that all of the subfractions have higher antimalarial activity than the parent extract or fraction.

Cytotoxicity of compounds can be categorized as non-toxic to mammalian cells if the IC50 >30 μg/mL⁻¹ (Nondo et al., 2017). Based on this criteria, all of the subfractions tested showed non-toxic. The IC50 value of the all of fractions >30 μg/mL⁻¹ ranging from 408.80 to >1000 (Table 3). The all of fractions also showed high selective as demonstrated with the SI value ranged from 131.93 to >125,000 (Table 3). The highest selectivity was obtained from the SF.3 with IC50 value >125,000 μg/mL⁻¹. Base on the SI value, the SF.3 is the most potential fractions to be isolated its active antimalarial compounds.

The antimalarial activity of Indonesian medicinal plants have been reported in the previous studies. The Eurycoma longifolia (pasak bumi) extracts were reported to have antimalarial activity with IC50 value ranged 2.21-19.02 μg/mL⁻¹ (Sholikhah et al., 2018). In addition, Lyles et al. (2014) reported antimalarial activity of synthetic compounds containing benzophenones and xanthones of edible fruit from Garcinia species with IC50 value >2 μg/mL⁻¹. The antimalarial activity of the subfractions tested in this study was higher than that from the two medicinal plants previously reported. It was indicated that the active subfractions of the H. aculeatus is very promising to be further explored for its antimalarial active compounds.

Phytochemical studies of H. aculeatus are very limited. The antimalarial active compounds of this plant have not been isolated and identified. In the previous studies, flavonoids contain were reported in the methanol extract and the active fraction (Turalely et al., 2011; 2012). In this study, the flavonoids were also identified in the SF.3. It is indicated that the flavonoids may be responsible for the antimalarial activity of this subfraction. The antimalarial activity of flavonoids isolated from medicinal plants was reported. Tetrahydroxyxanthone was reported to have antimalarial activity (Ignatuschkeno et al., 2000), whereas Lyles et al. (2014) also reported in vitro antimalarial activity of another xanthones from another plant.

CONCLUSION

The four subfractions of H. aculeatus leaves tested have high in vitro antimalarial activity and selectivity against P. falciparum. Further study will be focused to isolate and identify active antimalarial compounds from another subfraction.

ACKNOWLEDGMENT

The study was supported by Universitas Gadjah Mada, Yogyakarta through Thesis Recognition Program. We would like to thank Mrs. Rumbiati and Mr. Purwono from Departament of Parasitology, Mr. Wagiman and Mrs. Mosa from Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada for valuable assistants during the study. We also would like to thank Prof. Dr. Peter Protosch and Dr. Rini Muharini from Heinrich Heine Universitaet, Duessedorf, Germany for their suggestions.

REFERENCES

Fairhurst RM, 2016. Purpose of review—The emergence of artemisinin resistance in Southeast Asia, where artemisinin combination therapies (ACTs) are beginning...


