Indonesian Journal of Pharmacy

VOL 31 (1) 2020: 51–55 | SHORT COMMUNICATION

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

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Info Article

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

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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 IC_{50}, calculated using probit analysis with SPSS 16 for windows. The results showed that the four subfractions tested have a high antiplasmodial activity with IC_{50} values of 0.09; 0.18; 0.01; and 0.77 $\mu g.mL^{-1}$, respectively. In addition, these subfractions had IC_{50} values of >400 $\mu g.mL^{-1}$ 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 antiplasmodial 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* antiplasmodial 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 antiplasmodial 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 Boerl (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* antiplasmodial 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 $FeCl_3$ 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 ¾ 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* antiplasmodial activity and cytotoxicity.

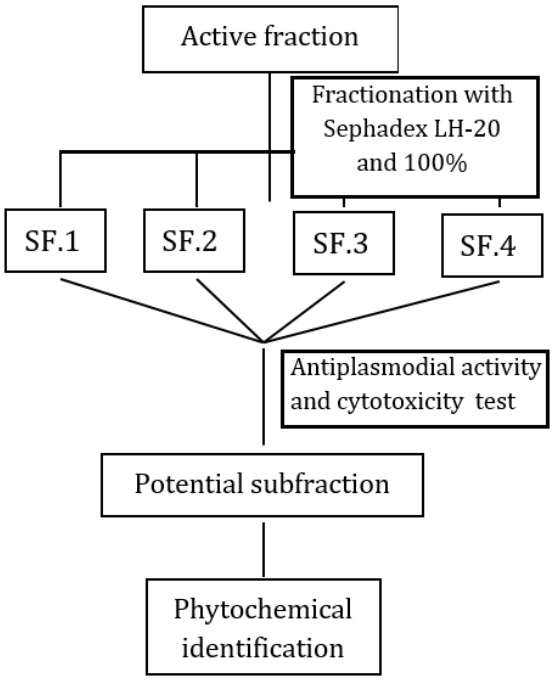

Figure 1. Scheme of the experimental procedure

In vitro antiplasmodial activity assay

In vitro antiplasmodial activity of each sufraction was tested against the chloroquine-resistant *P. falciparum* strain (FCR_3) 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 $\mu g.mL^{-1}$, depend on each fraction, were prepared using culture medium. One hundred µL of the sufraction 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 CO_2 incubator. The wells containing culture medium without subfraction were used as negative control. Followed after incubation, a thin blood smear of

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

No.	Subfraction	Form	% w/w	Rf	Color		
					Visible light	UV (λ 254 nm)	UV (λ 366 nm)
1	SF.1	Greenish white powder	0.39	0.86; 0.97	-	Green fluorescent	Blue fluorescent
2	SF.2	White powder	38.67	0.88	-	Green fluorescent	Blue fluorescent
3	SF.3	Yellowish white powder	37.50	0.89	-	Green fluorescent	Blue fluorescent
4	SF.4	Greenish powder	23.44	0.79; 0.89	-	Green fluorescent	Blue fluorescent

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

Subfraction 1 (SF.1)		Subfraction 2 (SF.2)		Subfraction 3 (SF.3)		Subfraction 4 (SF.4)	
Conc. (µg/mL)	Inhibition (%)	Conc. (µg/mL)	Inhibition (%)	Conc. (µg/mL)	Inhibition (%)	Conc. (µg/mL)	Inhibition (%)
60	94.89±2.87	100	78.46±15.68	20	92.05±2.93	20	85.6±3.55
50	94.54±3.98	50	68.97±9.09	10	74.89±4.76	10	65.22±2.72
25	91.57±3.54	10	61.25±7.38	5	67.09±2.21	5	63.14±4.21
20	86.36±12.33	5	58.87±13.97	1	66.14±5.54	0.5	58.61±5.12
10	73.01±4.83	1	55.23±5.85	0.5	64.67±3.00	0.05	54.37±3.45
5	62.89±2.76	0.5	52.89±8.18	0.1	63.57±2.66	0.005	42.48±5.36
1	57.61±8.40	0.1	46.85±3.57	0.05	62.73±4.94		
0.5	53.53±12.26	0.05	44.67±9.11	0.01	49.86±11.81		
0.1	49.88±4.56	0.01	41.46±6.96				
0.05	42.68±13.86	0.005	39.15±10.71				
IC_{50} (µg.mL^{-1})	**0.22**		**0.57**		**0.01**		**0.04**

each wells 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% (IC_{50}) 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 CO_2 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 IC_{50} 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 $FeCl_3$ spray reagents.

RESULT AND DISCUSSION

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

chloroform-ethyl acetate. The form and TLC profile of the subfractions (Table I). The inhibition of Plasmodium growth and *in vitro* antiplasmodial activity (IC_{50}) 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*

Subfraction	IC_{50} on Vero cells (µg/mL)	Selectivity Index
SF7.1	>700	>7692.31
SF7.2	408.80	2271.11
SF7.3	>1000	>125,000
SF7.4	1022.44	131.93

Antiplasmodial activity of natural products or synthetic compounds can be categorized into high if the IC_{50} value <5 µg.mL^{-1}; promising if the IC_{50} value between 5-15 µg.mL^{-1}, moderate if the IC_{50} value between 15-50 µg.mL^{-1} and, not active if the IC_{50} value >50 µg.mL^{-1} (Jonville *et al.,* 2008). Based on this criteria, all of the subfractions tested showed high antiplasmodial activity with the IC_{50} value <5 µg.mL^{-1} (Table 2). The highest antiplasmodial activity was obtained from the SF.3 with IC_{50} value of 0.01 µg.mL^{-1}. Furthermore, all of the subfractions had IC_{50} value lower than it's the 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 the all of the subfractions have higher antiplasmodial activity than the parent extract or fraction.

Cytotoxicity of compounds can be categorized 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 IC_{50} 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 IS value ranged from 131.93 to >125,000 (Table 3). The highest selectivity was obtained from the SF.3 with IC_{50} value >125,000 µg.mL^{-1}. Base on the SI value, the SF.3 is the most potential fractions to be isolated its active antiplasmodial compounds.

The antiplasmodial activity of Indonesian medicinal plants have been reported in the previous studies. The *Eurycoma longifolia* (*pasak bumi*) extracts were reported to have antiplasmodial activity with IC_{50} value ranged 2.21-19.02 µg.mL^{-1} (Sholikhah *et al.*, 2018). In addition, Lyles *et al.* (2014) reported antiplasmodial activity of a mixture of benzophenones and xanthones of edible fruit from *Garcinia* species with IC_{50} value >2 µg.mL^{-1}. The antiplasmodial 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 antiplasmodial 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 indicted that the flavonoids may be responsible for the antiplasmodial activity of this subfraction. The antiplasmodial activity of flavonoids isolated from medicinal plants was reported. Tetrahydroxyxanthone was reported to have antimalarial activity (Ignatushchenko *et al.,* 2000), whereas Lyles *et al.* (2014) also reported *in vitro* antiplasmodial activity of another xanthones from another plant.

CONCLUSION

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

ACKNOWLEDEGMENT

The study was supported by Universitas Gadjah Mada, Yogyakarta through Thesis Recognition Program. We would like to thank Mrs. Rumbiwati and Mr. Purwono from Departement 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 Proksch and Dr. Rini Muharini from Heinrich Heine Universitaet, Duessedorf, Germany for their suggestions.

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