INTRODUCTION

Malaria is one of the most important health problems in sub-tropical and tropical countries. The World Health Organization estimates that 2,300 million people, or 41% of the total world population, live in areas with malaria risk. More than 300 to 500 million clinical cases are reported annually resulting in at least 1.5 to 2.7 million deaths. Approximately 1 million deaths among children under 5 years old are attributed to malaria alone or in combination with other diseases (WHO, 1997, 1998). In Indonesia alone, 87 million people live in areas at risk for malaria and it is estimated that 6 million cases are reported annually. The National Household Health Survey of 1995 estimated that 32,000 deaths, or 2% of deaths, were caused by malaria annually. A significant increase in malaria occurred during 1997-2001 due to the monetary crisis in Indonesia. During that time, the incidence of malaria increased significantly each year, becoming 0.62 per 1,000 people with an annual...
incidence rate in Java-Bali in the region of 0.12-11.73 (Achmadi, 2003).

Choloquine is still recommended as the first line drug for the treatment of chloroquine-sensitive Plasmodium malaria in Indonesia, while sulfadoxin-pyrimethamine is used as a second line drug against chloroquine-resistant Plasmodium malaria. However, the resistance of Plasmodium species, especially P. falciparum, to chloroquine has been reported in certain provinces of Indonesia since 1970 (Dondero et al, 1974; Ebisawa and Fukuyama, 1975; Baird et al, 1996; Fryauff et al, 1998). The antimalarial resistance of Plasmodium has initiated numerous studies aimed at identifying new antimalarial agents.

One of the strategies in the search for new antimalarial compounds is a study of active constituents of medicinal plants. In malaria endemic areas of Indonesia, medicinal plants, such as makasar fruit (Brucea javanica (L.) Merr.), papaya leaves (Carica papaya Linn.), pasak bumi roots (Eurycoma longifolia Jack.), mahoni leaves (Swietenia mahagoni Jacq.), mimba leaves (Azadirachta indica Juss.), pule seeds (Alstonea scolaris) and meniran herb (Phyllanthus niruri L.) are often used to treat malaria (Anonymous, 1990; Sudarsono et al, 1996). However, scientific information about the antimalarial activity of these plants is very limited. It is important, therefore, to investigate the antimalarial activities of these medicinal plants in order to determine their potential as sources of new antimalarial agents.

Phyllanthus niruri, locally named meniran, is one of the medicinal plants traditionally used to treat malaria in Indonesia. Phyllanthus niruri has been used traditionally to treat various illnesses, including renal stones, gastrointestinal disturbances, cough, hepatitis, gonorrhea, fever and malaria. This plant was reported to possess hypoglycemic activity (Hukuri et al, 1988), angiotensin-converting enzyme inhibition (Ueno et al, 1988), lipid lowering activity (Khanna et al, 2002), anticancer activity (Giridharan et al, 2002) and anti-HIV activity (Qian-Cutrone et al, 1996). However, very little scientific information is available about its activity against P. falciparum although this plant is extensively used to treat malaria.

In our attempt to find new natural compounds with antimalarial activity that may provide an alternative to chloroquine, we report here on the in vitro antiplasmodial activity and cytotoxicity of extracts of P. niruri herb. In this preliminary study, chloroformic, methanolic and aqueous extracts of P. niruri were evaluated for its antiplasmodial activity against P. falciparum and cytotoxicity of these extracts was investigated on the HeLa cell line. For the most active extracts, in vivo antiplasmodial activity in P. berghei infected mice was also investigated.

MATERIALS AND METHODS

Plant extracts

The plant P. niruri was collected in its natural habitat in Sleman, Yogyakarta and identified by comparison with reference specimens in the Laboratory of Pharmacognocy, Faculty of Pharmacy, Gadjah Mada University (GMU), Yogyakarta. The herb was air-dried and ground to provide a fine powder. Extracts were then prepared by maceration of the powder with chloroform, methanol and distilled water, sequentially. Two hundred grams of the powder was macerated with 1,000 ml of chloroform for 24 hours. After stirring for 3 hours, the chloroform was separated by filtration and then maceration was repeated three times on the residue. The three macerates were pooled and concentrated by a rotary evaporator to obtain a chloroform extract. The residue left was remacerated with methanol then distilled water in the same manner as maceration was performed with chloroform. Upon evaporation under reduced pressure, chloroformic, methanolic and aqueous extracts were obtained.
Parasite strains and in vitro culture

The FCR-3 strain of *P. falciparum*, chloroquine-resistant with an IC\textsubscript{50} > 150 ng/ml and D-10 chloroquine-sensitive strain with an IC\textsubscript{50} < 50 ng/ml were cultured continuously according to Trager and Jensen (1978) with modifications described by Van Huyssen and Rieckmann (1993). The parasites were maintained in vitro in human erythrocytes (O\textsuperscript{+}), diluted to 1% hematocrit in RPMI 1640 (Sigma) supplemented with 25 mM HEPES and 30 mM NaHCO\textsubscript{3} and complemented with 5% human O\textsuperscript{+} serum. Parasite cultures were incubated at 37°C in candle jars with a daily change of medium. Parasite cultures were synchronized with 5% D-sorbitol given every 48 hours as reported by Lambros and Vanderberg (1979).

Parasite and mice strains for in vivo antiplasmodial assay

The Swiss mice were bred at the Department of Pharmacology and Toxicology, Faculty of Medicine GMU, Yogyakarta. The ANKA strain of *P. berghei* was obtained from the Department of Parasitology, Faculty of Medicine GMU, Yogyakarta, Indonesia.

Assay for in vitro antiplasmodial activity

The antiplasmodial activity of plant extracts on the two strains of *P. falciparum* was evaluated according to the semiautomatic microdilution technique of Desjardins et al (1979). Each extract was tested in triplicate in three independent experiments. Testing was performed in 96-well culture plates with culture mostly at the ring stage at 0.5-1% parasitemia (hematocrit, 1%). One hundred µl of parasite culture was distributed into each well plate and 100 µl of culture medium containing extracts at various concentrations was added. The parasite cultures and extracts were then incubated for 24 and 72 hours before adding [\textsuperscript{3}H]-hypoxanthine (0.25 µCi per well). Following incubation, the parasites were harvested onto fiber glass filters using an automatic cell har-
daily for 4 consecutive days, beginning on the
day of infection, starting two hours after in-
oculation until day 3. The level of parasitemia
was determined the day following the last
treatment. The ED_{50}, which is the dose lead-
ning to 50% parasite growth inhibition com-
pared to growth in the control, was evaluated
from a plot of activity (expressed as a percent-
age of the activity in the control) versus the
log dose. These experiments were conducted
in accordance with the Experimental Animal
Guidelines of Laboratory Method on Toxicol-

**Statistical analysis**

Comparison of antiplasmodial activity
(IC_{50}s) among three extracts tested after 24
hours and 72 hours of incubation on the two
*P. falciparum* strains was performed using the
Kruskal Wallis test, followed by the Mann-
Whitney U test.

**RESULTS**

The IC_{50} values obtained with extracts of
various plants against FCR-3 and D-10 strains
using the radioactive method are summarized
in Table 1. Data are expressed as the median
and range. The IC_{50} ranged from 2.3 to 200.4
µg/ml. Among the three extracts tested in the
present study, the methanolic extract showed
highest antiplasmodial activity in comparison
with other extracts tested with IC_{50} values
ranging from 2.3 to 3.9 µg/ml. The IC_{50} values
were significantly different (p < 0.05) compared
to the chloroformic extract (IC_{50} ranged from
132.6 to 200.4 µg/ml). However, no significant
differences were observed (p > 0.05) when they
were compared with aqueous extract (IC_{50} ranged from 2.9 to 4.1 µg/ml).

When the two incubation times are com-
pared, two kinds of results are observed. For
the aqueous and methanolic extracts, no cu-
mulative effect was observed. The IC_{50} values
of these two extracts 24 hours after contact
between the parasites and extracts were not
significantly different (p > 0.05) compared to
72 hours after contact. In contrast, on the
tested chloroformic extract, the IC_{50} values 72
hours after contact revealed a cumulative ef-
fect expressed by the lower 72-hour IC_{50} val-
ues than the 24-hour values (p < 0.05).

In regard to chloroquine sensitivity of the
*P. falciparum* strain with the aqueous and IC_{50}
for the chloroquine-resistant (FCR-3 strain)
and -sensitive (D-10) strains were similar (p >
0.05). Thus it appears the potential antima-

<table>
<thead>
<tr>
<th>Strain/cell</th>
<th>FCR-3 strain</th>
<th>D-10 strain</th>
<th>HeLa cell</th>
<th>CI^{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>24-hour</td>
<td>72-hour</td>
<td>24-hour</td>
<td>72-hour</td>
</tr>
<tr>
<td>Aqueous</td>
<td>4.1</td>
<td>3.3</td>
<td>3.4</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>(3.6-4.5)</td>
<td>(2.7-3.9)</td>
<td>(2.8-3.9)</td>
<td>(2.5-3.3)</td>
</tr>
<tr>
<td>Methanolic</td>
<td>4</td>
<td>2.3</td>
<td>3.2</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>(2.7-5.2)</td>
<td>(1.8-2.7)</td>
<td>(2.2-3.4)</td>
<td>(1.9-3.1)</td>
</tr>
<tr>
<td>Chloroformic</td>
<td>202.3</td>
<td>166.5</td>
<td>161.2</td>
<td>132.5</td>
</tr>
<tr>
<td></td>
<td>(192.1-212.5)</td>
<td>(155.8-177.1)</td>
<td>(147.6-174.8)</td>
<td>(120.0-145.0)</td>
</tr>
</tbody>
</table>

^{a}median (range); ^{b}CI, Cytotoxicity Index: IC_{50} against Hela cells/IC_{50} against FCR-3 strain.
Plasmodial activity and cytotoxicity of *Phyllanthus niruri* L.

Table 2

*In vivo* antiplasmodial activity (ED₅₀ in mg/kg/d) of extracts for meniran (*P. niruri*) herb against *P. berghei* infected mice.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Dose tested (mg/kg/d)</th>
<th>Parasitemia (%)</th>
<th>Growth inhibition (%)</th>
<th>Mortality (n/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>50</td>
<td>42 ± 7</td>
<td>77.2 ± 11.7</td>
<td>0/6</td>
</tr>
<tr>
<td>Estimated ED₅₀ : 20.0 mg/kg BW/d</td>
<td>100</td>
<td>42 ± 6</td>
<td>78.2 ± 11.5</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>43 ± 5</td>
<td>79.0 ± 12.6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>51 ± 3</td>
<td>95.1 ± 6.0</td>
<td>2/6</td>
</tr>
<tr>
<td>Methanolic</td>
<td>12.5</td>
<td>41 ± 6</td>
<td>75.2 ± 13.8</td>
<td>0/6</td>
</tr>
<tr>
<td>Estimated ED₅₀ : 9.1 mg/kg BW/d</td>
<td>25</td>
<td>34 ± 11</td>
<td>62.9 ± 19.6</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>52 ± 8</td>
<td>95.8 ± 14.6</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>51 ± 4</td>
<td>93.9 ± 7.6</td>
<td>2/6</td>
</tr>
<tr>
<td>Controlᵃ</td>
<td>-</td>
<td>54 ± 15</td>
<td>100</td>
<td>0/6</td>
</tr>
</tbody>
</table>

ᵃRPMI; bMortality is defined as n/N, where n is the number of dead mice and N is the number of mice in each group.

Plasmodial compounds contained in these extracts may have interfered with *P. falciparum* growth by a different mechanism than that of chloroquine. However, for the chloroformic extract, the IC₅₀ was higher for the chloroquine-resistant strain than for chloroquine-sensitive strain. The extracts of *P. niruri* also showed different levels of cytotoxicity (Table 1). The IC₅₀ values of the various extracts of this plant on HeLa cells ranged from 111.0 to > 200 µg/ml depending on the kind of extract and incubation time. The methanolic extract exhibiting the highest antiplasmodial activity was also more toxic (median IC₅₀, 167.7 µg/ml after 24 hours and 134.1 µg/ml after 72 hours) than the aqueous extract after 24-hour incubation (IC₅₀, 435.7 µg/ml) but it was less toxic after 72-hour incubation (IC₅₀, 99.2 µg/ml) (p < 0.05). The mean Cytotoxicity Index (CI = IC₅₀ against HeLa/IC₅₀ against the FCR-3 strain) of methanolic extract after 24-hour incubation (median CI, 41.3) was lower than that of the aqueous extract after 24-hour incubation (CI, 106.8) but was higher after 72-hour incubation (CI, 31.0). Inversely, in comparison with chloroformic extract (IC₅₀, 111.0 µg/ml after 24 hours and 262.9 µg/ml after 72 hours), the methanolic extract was less toxic by 24-hour incubation but more toxic by 72-hour incubation (p < 0.05). However, the mean CI of the methanolic extract was higher than the chloroformic extract, they are measured both at 24-hour (CI, 0.5) and 72-hour incubation (CI, 1.6) (p < 0.05).

Of the three extracts tested in the in vitro study, two extracts (methanolic and aqueous) displayed strong antiplasmodial activity. These two extracts were then evaluated for their in vivo antiplasmodial activity on *P. berghei* infected mice. The results of this study show the methanolic extract was more active in vivo (ED₅₀ = 9.1 mg/kg BW/d) than the aqueous extract (ED₅₀ = 20.0 mg/kg BW/d) as shown in Table 2. However, this methanolic extract was not well tolerated in mice compared with the aqueous extract, since loss was observed by 4 days of treatment at 50 mg/kg BW/d.

**DISCUSSION**

The IC₅₀ values obtained for the aqueous and methanolic extracts of this plant are interesting when they were compared with the results from other plant extracts reported in
the literature. *Artemisia annua* (the source of artemisinin) and *Azadirachta indica* (Neem) have an IC$_{50}$ of 3.9 µg/ml and 2.3 to 12.5 µg/ml, respectively (O’Neill et al, 1985; Benoit-Vical et al, 1996). Both plants are considered as reference medicinal plants by numerous authors due to their wide use in the treatment of malaria (Benoit-Vical et al, 1996).

Gessler et al (1994) recommended if the extract displayed an IC$_{50}$ less than 10 µg/ml, antiplasmodial activity was very good, from 10 to 50 µg/ml, the antiplasmodial activity was moderate and over 50 µg/ml the extract was considered to have low activity. Based on this recommendation, the aqueous and methanolic extracts of *P. niruri* with IC$_{50}$ values from 2.9 to 4.1 µg/ml and from 2.3 to 4.0 µg/ml, respectively, can be concluded as having very good antiplasmodial activity. These results suggest more active compounds may be extracted with a polar solvent and should be evaluated for in vivo antiplasmodial activity. For this purpose, a 4-day suppressive test was performed on male Swiss mice using *P. berghei*.

The results summarized in Table 2 show the methanolic and aqueous extracts were active in vivo with ED$_{50}$ of 9.1 and 20.0 mg/kg BW/d, respectively. The in vivo antiplasmodial activity can be classified as moderate, good and very good activity if the extract displays a percent growth inhibition equal to or greater than 50% at a dose of 500, 250 and 100 mg/kg BW/d, respectively (Munoz et al, 2000). Base on this classification, the two tested extracts exhibited very good antiplasmodial activity. These results are consistent with the values obtained from the in vitro antiplasmodial study.

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