IN VITRO ANTIPLASMODIAL ACTIVITY OF COUMARIN 8-HYDROXYISOCAPNOLACTONE-2′,3′-DIOL ISOLATED FROM Micromelum minutum (G. Forst.) Wight & Arn

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ABSTRACT
Malaria remains a health problem in tropical and subtropical countries. The spread of antimalarial resistant parasites has prompted the research to find more effective new antimalarials. One strategy to discover new antimalarial drugs is through the exploration of medicinal plants traditionally used to treat malaria. Micromelum minutum is one of medicinal plants that has been used to treat malaria and other infectious diseases in Malaysia. The coumarin 8-hydroxyisocapnolactone-2′,3′-diol has been isolated from this plant and showed to have strong cytotoxic activities. This study aimed to evaluate the in vitro antiplasmodial activity of 8-hydroxyisocapnolactone-2′,3′-diol against chloroquine-resistant (FCR-3) and chloroquine-sensitive (D-10) Plasmodium falciparum. Culture of P. falciparum was continuously grown using a candle jar. Antiplasmodial assay was conducted by microradioactive method. The antiplasmodial activity was expressed by the IC50 indicating the concentration of the compound yielding 50% inhibition of the parasite growth. The results showed that 8-hydroxyisocapnolactone-2′,3′-diol has an in vitro antiplasmodial activity against FCR-3 and D-10 with the IC50 values of 6.39 μg/mL (16.99 μM) and 24.23 μg/mL (64.45 μM), respectively.

Key words: 8-hydroxyisocapnolactone-2′,3′-diol, M. minutum, antiplasmodial activity, P. falciparum, FCR-3, D-10

INTRODUCTION
Malaria remains a serious public health problem in tropical and subtropical countries. In 2010, it was estimated that 219 million cases of malaria occurred worldwide and lead to 660,000 deaths (CDC, 2012). According to the World Health Organization (WHO), in 2011 approximately 3.3 billion people live in areas with high risk of malaria (WHO, 2012). In Indonesia, it was estimated that 45% of the population live in areas at risk of malaria transmission (Ministry of Health of the Republic of Indonesia, 2010). Almost all provinces in Indonesia have malaria-endemic areas with different level of endemicity. Areas with high malaria endemicity are located outside the Java island, such as Maluku, Papua, Nusa Tenggara, Sulawesi, Borneo and Sumatra. In 2009 1,100,000 cases of clinical malaria have been reported in Indonesia and it increased to 1,800,000 cases in 2010 (Ministry of Health of the Republic of Indonesia, 2011).

Although malaria eradication program has been conducted to eliminate malaria, however its prevalence remains high. Antimalarial drug resistance to Plasmodium has emerged as one of the cause of malaria eradication failures. The extensive and rapid spread of parasites resistance to antimalarials, especially chloroquine, and the limited antimalarial drugs in the market have driven the need to find new antimalarial drugs. One of strategies in new antimalarials discovery is through the exploration of medicinal plants traditionally used to treat malaria.

Figure 1. 8-Hydroxyisocapnolactone-2′,3′-diol
Micromelum minutum has been used to treat malaria as well as other infectious diseases
in Malaysia (Burkill, 1966). The coumarin 8-hydroxyisocapnolactone-2’3’-diol (Figure 1) has been isolated as the main constituent of this plant that grows in Sabah, Malaysia (Rahmani et al., 2003). This compound has been reported to have strong cytotoxic activity against CEM-SS, HL60, HeLa, HepG2, and NS-1 cancer cell lines (Susidarti et al., 2009; Yasmina et al., 2005). However, the antiplasmodial activity of this compound has never been studied, yet. This study aimed to evaluate the in vitro antiplasmodial activity of 8-hydroxyisocapnolactone-2’3’-diol against chloroquine-resistant (FCR-3) and chloroquine-sensitive (D-10) P. falciparum.

**MATERIAL AND METHODS**

8-Hydroxyisocapnolactone-2’3’-diol used in this study was obtained from Dr. Ratna Asmah Susidarti, Faculty of Pharmacy, Gadjah Mada University. The compound was isolated from the chloroform extract of the leaves of *M. minutum* by Rahmani et al. (2003). Strains of *P. falciparum* FCR-3 (chloroquine-resistant) and D-10 (chloroquine-sensitive) were obtained from the Eijkman Institute for Molecular Biology, Jakarta. Other chemicals used in the antiplasmodial assay were RPMI 1640 (Gibco), HEPES (Sigma), gentamicin (Merck), NaHCO3 (Sigma), sorbitol (Merck), Giemsa (Sigma), sterile aquabidest, NaOH 0,1 M, human serum group O+, human blood cells group O+, ethanol 70% (technical grade), methanol, (2,8-3H)hypoxanthine isotope (Sigma) and betafluor for scintillation (Packard).

**Continuous culturing of *P. falciparum***

Ampoules containing *P. falciparum* FCR-3 and D-10 strains were removed from liquid nitrogen, placed in water bath at 37°C until the blood was fully thawed and then transferred to conical tube. As many as 0.2mL of 12% NaCl solution was added drop by drop for every 1mL thawed blood. The solution was mixed and allowed to stand for 3min. Ten milliliters of 1.6% NaCl solution was added drop by drop for every 1mL of thawed blood. The solutions was centrifuged at 1500rpm for 5min and then the supernatant was removed. Ten milliliters of a mixture of 0.9% NaCl and 0.2% dextrose solution was added for each 1mL thawed blood. This solution was centrifuged again at 1500rpm for 5min and the supernatant was removed. The precipitated cells were slowly resuspended in complete medium containing human serum and then incubated for 48h.

**Culturing *P. falciparum***

*Plasmodium falciparum* was cultured using a candle jar method developed by Trager and Jensen (1976). Parasite-infected erythrocytes were cultured in a culture flask containing 10mL of complete medium (containing 10% serum) to a final hematocrit of 1.5%. Manipulation of the culture was performed in a laminar air flow cabinet under aseptic conditions. The *Plasmodium* culture was then placed in the CO2 incubator at 37°C.

**Maintaining culture***

The *Plasmodium* culture was maintained by replacing the media every 24h of incubation. When the parasitemia was too high (>10%), subculture should be made by adding normal red blood cells until the parasitaemia <1%.

**Sample Preparation***

Five milligrams of 8-Hydroxyisocapnolactone-2’3’-diol was dissolved in 100µL of DMSO and added to RPMI 1640 medium to obtain the volume of 5.0mL as a stock solution. This solution was then diluted with RPMI 1640 to obtain a series of concentrations of 10; 25; 50; 100; and 150µg/mL.

**Synchronization***

Malaria parasites undergo various stages of growth within erythrocytes, namely young trophozoit (ring stage), trophozoit, and finally schizont. For antiplasmodial activity testing, the ring stage parasites were used after synchronized with 5% sorbitol solution. Parasites were centrifuged at 1500rpm for 10min. Supernatant was removed and the precipitate was soaked in a sterile 5% sorbitol solution as many as 5 x volume of supernatant and then allowed to stand at room temperature for 10min. During this period, schizont stage parasites have been lysed and leave the trophozoit stage. With additional of 5 x volume of complete medium, the parasite was then
washed by centrifugation at 1500 rpm for 10 min. The precipitated parasite that only consists of a ring stage was obtained. The parasite was then returned to the culture flask. Synchronization was carried out every 48 h to obtain the more homogeneous ring-stage parasites.

**Preparation of microcultures and antiplasmodial activity assay** (Rieckman et al., 1978).

The 96 wells microplate was used to culture the parasites. Each well was filled with 100 µL of complete medium containing parasites with 1.5% hematocrit and 1% parasitemia. The test solutions were prepared in sterile bottles, and by using a 100 µL micropipette, each solution was transferred into the wells ranging from low to high concentrations, whereas 100 µL RPMI 1640 solution was added into the untreated group. Each concentration was made triplicate. Microculture plate was then placed in a vacuum desicator containing a candle in it (candle jar). The desiccator was covered when the candle flame almost die, so that the optimal concentration of gas was available for the culture. The jar was then incubated in a CO₂ incubator at 37°C for 60 h.

**Examination of microradioactive methods** (Desjardins et al., 1979).

Preparation of (2,8-³H) hypoxanthine solution

(2,8-³H) hypoxanthine was used as a marker of parasite growth. The amount of isotopes used by parasites can be measured that represented the magnitude of parasite growth in culture. The isotope was supplied in ampoules containing 1.0 mCi. A total of 20 µL isotope was diluted with RPMI 1640 containing 10% human serum to a volume of 4.0 mL to obtain a solution that has a radioactivity of 0.25 µCi per 50 µL.

Addition of hypoxanthine isotopes into microcultures

After 60 h incubation period and the culture growth healthily without contamination, 50 µL hypoxanthine isotope was added to each well. The culture was homogeneously mixed by shaking it on the table, then returned to the candle jar and incubated at 37°C for 12 h to obtain a 72 h incubation period.

Harvesting parasites and counting in β counter

Harvester was arranged so that the radioactive content of each well will be caught in the strainer filters. The filter was previously moistened with 0.9% saline solution. This filter was then washed with sterile aquabidest for 20 s to lyse erythrocytes and wash the hemoglobin. The filter was then dried in an oven at 60°C. The sphere part of the filter was moved into counting vials. Those vials was then filled with 0.5 mL of liquid scintillation betafluor and put in a Liquid Scintillation Analyzer. Counting efficiency is approximately 30%. The Print out of the isotope usage from β counter calculation result was read on three different zones and expressed in counts per minute (cpm). The number of isotopes used represented the growth of parasite. The percentage of parasite growth inhibition can be calculated by the following formula:

\[
\%_{\text{inhibition}} = \frac{\text{cpm control} - \text{cpm sample}}{\text{cpm control}} \times 100\%
\]

**Analysis**

The result was shown as the relationship curve between the concentration of 8-hydroxyisocapnolactone-2',3'-diol and % inhibition of *Plasmodium* growth. Inhibitory Concentration fifty percent of parasite growth (IC₅₀) value was determined by probit analysis according to the relation of log concentration of test compound and % inhibition of parasite growth.

![Figure 2. Structure of (2,8-³H)hypoxanthine isotope](image)

**RESULT AND DISCUSSION**

*Plasmodium falciparum* proliferation requires large amounts of purine. In the absence of de novo purine biosynthesis in the parasite, hence the need of purines was obtained from the environment. Purine bases released by the hydrolytic degradation of nucleic acids and nucleotides can be salvaged.
Hypoxanthine is a precursor of purine salvage pathway. In the Plasmodium cell, hypoxanthine will be metabolized into inosine monophosphate (IMP). Through a series of chemical and enzymatic processes IMP will be converted to RNA and DNA adenine and guanine bases (Berrens et al., 1995). The existence of a large number of (2,8-\textsuperscript{3}H)hypoxanthine isotopes (Figure 2) and the presence of a small number of purine bases and nucleosides in the medium, a number of isotopes will be taken and metabolized by the parasite. Therefore, by measuring the radioactivity of (2,8-\textsuperscript{3}H) hypoxanthine isotopes which have been taken by parasites, the number of alive parasites can be determined.

Treatment of 1, 5 and 50\(\mu\)g/mL 8-hydroxyisocapnolactone-2’3’-diol on chloroquine-resistant \textit{P. falciparum} (FCR-3) showed a dose-dependent profile in all three measurement zones (Table I). Observation on the three zones showed that the percentage of growth inhibition of the parasites was increased along with the increasing levels of the test compound. The same phenomenon was also observed on the D-10 strain treated with 25 and 50\(\mu\)g/mL of the coumarin (Table II). The graph showing the relationship between the concentration of test compound with the average of % growth inhibition of \textit{P. falciparum} FCR-3 and D - 10 can be seen in Figure 3 and 4, respectively.

Antiplasmodial activity was expressed as the IC\textsubscript{50} value and determined by probit analysis. The IC\textsubscript{50} values of 8-hydroxyisocapnolactone-2’3’-diol against \textit{P. falciparum} D-10 and FCR-3 strains in all three measurement zones can be seen in Table III. The 8-hydroxyisocapnolactone-2’3’-diol was found to be significantly active against chloroquine-resistant and sensitive strains of \textit{P. falciparum} with IC\textsubscript{50} of 6.39\(\mu\)g/mL (16.99\(\mu\)M) and 24.23\(\mu\)g/mL (64.45\(\mu\)M), respectively. The antiplasmodial activity of 8-hydroxyisocapnolactone-2’3’-diol against chloroquine-resistant strain was stronger than chloroquine-sensitive strain. Based on this finding, this compound was expected to have different mechanism of action with chloroquine. The mechanism of action of chloroquine as antiplasmodial related to its ability to inhibit polymerization and detoxification of heme, the ability to bind and

### Table I. Inhibition of the growth (%) of chloroquine-resistant \textit{P. falciparum} (FCR-3) due to administration of 8-hydroxyisocapnolactone-2’3’-diol on the three measurement zones

| Concentration (\(\mu\)g/mL) | Zona 0-3 keV (A) | Zona 0-5 keV (B) | Zona 0.5-8 keV (C) | mean ± SD  |
|-----------------------------|-----------------|-----------------|-------------------|-----------|
| 50                          | 98.76           | 98.83           | 98.81             | 98.80 ± 0.04 |
| 5                           | 21.64           | 21.87           | 22.15             | 21.89 ± 0.26 |
| 1                           | 11.72           | 13.06           | 14.21             | 13.00 ± 1.25 |
| 0.5                         | 9.00            | 14.25           | 17.10             | 13.45 ± 4.11 |
| 0.1                         | 7.13            | 7.96            | 8.70              | 7.93 ± 0.79  |
| 0.05                        | 8.88            | 10.30           | 10.87             | 9.99 ± 1.07  |

### Table II. Inhibition of the growth (%) of chloroquine-sensitive \textit{P. falciparum} (D-10) due to administration of 8-hydroxyisocapnolactone-2’3’-diol on the three measurement zones

| Concentration (\(\mu\)g/mL) | Zona 0-3 keV (A) | Zona 0-5 keV (B) | Zona 0.5-8 keV (C) | mean ± SD  |
|-----------------------------|-----------------|-----------------|-------------------|-----------|
| 150                         | 98.17           | 98.05           | 97.92             | 98.05 ± 0.12 |
| 100                         | 97.59           | 97.27           | 97.00             | 97.29 ± 0.29 |
| 50                          | 94.46           | 94.48           | 94.34             | 94.43 ± 0.07 |
| 25                          | 62.67           | 62.40           | 60.70             | 61.92 ± 1.07 |
| 10                          | 0.00            | 0.00            | 0.00              | 0.00       |
alter the nature of DNA, and the weak base properties of chloroquine allowed a high accumulation of this compound in Plasmodium cell (Ahmad and Sutanto, 2003).

8-Hydroxyisocapnolactone-2’3’-diol has the same basic chemical structure as 7,8-dihydroxycoumarin (daphnetin), but has a side chain attached to oxygen atom at C-7. The side chain consists of alkyl group containing 5 C atoms with 2 hydroxyl groups at C-2’ and C-3’ and a γ-lactone ring substituted by methylene at position 4” Daphnetin was first isolated from Daphne sp. The compounds with similar structure will usually shown a similar biological activity. Both of these coumarins have antiplasmodial activity. The IC_{50} values of daphnetin on P. falciparum in vitro was 4.45-7.12µg/mL (25-40µM). Daphnetin was able to decrease the activity of superoxide dismutase (SOD) by 60% and inhibited the synthesis of DNA on P. falciparum in vitro (Mu, et al., 2003). SOD is an enzyme that catalyze the reaction of the superoxide (O2^-) to hydrogen peroxide (H2O2) and oxygen (O2) in the presence of acid, according to the reaction of 2O2^- + 2H^+ → H2O2 + O2. This was a spontaneous reaction, but the reaction rate is stimulated by the presence of SOD. Superoxide together with hydrogen peroxide (H2O2), peroxy radicals (ROO•) and hydroxyl radical (OH•) were strong oxidator produced in the metabolism process in the cells. The oxygen has been played an important role in the injury or damage of the cells (Murray, 2003). Even though it seemed to be less dangerous than hydroxyl radical, but

Figure 3. Graph between the concentration of 8-hydroxyisocapnolactone-2’3’-diol versus % inhibition of the growth of chloroquine-resistant P. falciparum (FCR-3) on the three measurement zones (A=0.3 keV; B=0.5 keV; C=0.5-8 keV)

Figure 4. Graph between the concentration of 8-hydroxyisocapnolactone-2’3’-diol versus % inhibition of the growth of chloroquine-sensitive P. falciparum (D-10) on the three measurement zones (A=0.3 keV; B=0.5 keV; C=0.5-8 keV)
Superoxide was more reactive than hydrogen peroxide in physiologic system (Halliwell and Gutteridge, 1999). Inhibition of SOD resulted in increasing the amount of superoxide in the parasites. One other species, HO₂⁻, which was a protonated superoxide and slightly more reactive than superoxide itself. HO₂⁻ formed at acidic pH near the membrane may caused severe harm as a result of fatty acid peroxidation (Halliwell and Gutteridge, 1999). Superoxide in large quantities can lead to injury and even cells death (Murray, 2003).

Other study showed that daphnetin decrease the DNA synthesis of P. falciparum in vitro (Yang et al., 1992). DNA synthesis occurs in the mitotic process. During this process, all the nucleus DNA is replicated and then the cells was replicated. DNA is involved in the synthesis of protein where the genes in DNA control the synthesis of various types of RNA associated with the protein synthesis. The decrease in DNA synthesis, parasite replication is inhibited, so the parasites will die due to lack of proteins that play an important role in the metabolic processes in the parasite. The inhibition of DNA synthesis at various growth stages of P. falciparum by daphnetin has been carried out on the synchronized culture. The result showed that daphnetin inhibits DNA synthesis of P. falciparum on trophozoit stage. Based on the similarity in basic chemical structure with daphnetin, it was estimated that the mechanism of antimalarial activity of 8-hydroxyisocapno-lactone-2'-3'-diol containing substituted hydroxyl group at C-7 position still has good activity. The antimalarial activity of 8-hydroxyisocapno-lactone-2'-3'-diol against P. Falciparum provides a scientific evidence regarding the usefulness of M. minutum as an antimalarial. The result of this study is expected to be utilized in the development of antimalarial drugs.

**CONCLUSION**

8-Hydroxyisocapnolactone-2'-3'-diol isolated from M. minutum has in vitro antimalarial activity against P. falciparum FCR-3 and D-10 strains with IC₅₀ values of 6.39µg/mL (16.99μM) and 24.23mg/mL (64.45μM), respectively.

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