STAGE-DEPENDENT EFFECTS OF Quassia borneensis NOOT. (SIMAROUBACEAE) CHLOROFORM EXTRACTS ON ERYTHROCYTES INFECTED WITH CHLOROQUINE-RESISTANT *Plasmodium berghei* NK65

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Graphical abstract

Abstract

This study was conducted to screen the antiplasmodial activity of chloroform extracts of *Quassia borneensis* against different intraerythrocytic cycle of chloroquine-resistant *Plasmodium berghei* NK65. The antiplasmodial activity was measured using SYBR green I assay for determining the concentration that inhibited 50% (IC_{50}) of the *Plasmodium*'s activity after 24 hours of treatment (0.0001-100 mg mL^{-1}). All of the extracts showed potent activity against all of the cycles. The trophozoite and schizont stages were considerably more sensitive to the extracts than the ring-stages parasites. The values were compared to the controls treated with chloroquine and artemisinin. Hence, the chloroform extracts of roots and bark of *Q. borneensis* demonstrated potent activities towards each life cycle of chloroquine-resistant *P. berghei* and has a potential to be developed as an antimalarial agent.

Keywords: Q. borneensis, antimalarial, P. berghei, intraerythrocytes

Abstrak

Kajian ini dijalankan bagi menyaring aktiviti antiplasmodium ekstrak kloroform daripada *Quassia borneensis* terhadap kitaran intraeritrositik *Plasmodium berghei* NK65 yang berbeza. Aktiviti antiplasmodium telah diukur menggunakan asai SYBR green I bagi menentukan kepekatan yang boleh merencat 50% (IC_{50}) aktiviti *Plasmodium* selepas rawatan selama 24 jam (0.0001-100 mg mL^{-1}). Semua ekstrak menunjukkan aktiviti yang poten terhadap semua kitaran hidup parasit. Peringkat trofozoit dan skizon adalah lebih sensitif terhadap ekstrak berbanding peringkat cecincin. Bacaan telah dibandingkan dengan kawalan yang dirawat dengan klorokuin dan artemisinin. Justeru, ekstrak klorofom akar dan kulit *Q. borneensis* menunjukkan aktiviti perencatan yang poten bagi setiap peringkat kitaran hidup *P. berghei* dan berpotensi untuk dibangunkan sebagai agen antimalaria.

Kata kunci: Q. borneensis, antimalaria, P. berghei, intraeritrositik

1.0 INTRODUCTION

Over 300 million clinical cases of malaria occur annually, resulting in more than 1 million deaths [1]. Unfortunately, because of the prevalence of drug-resistant malaria parasite strains, existing drugs are increasingly losing their efficacies. Therefore, there is an urgent need to identify new drug leads to reduce the global malaria burden. In Malaysia, since 1995, figures showed that the incident of malaria has been declining from 296.7 to 48.3 in 1999. However, the mortality rate has been steadily increasing [2].

Simaroubaceae had been numerously reported for its potent biological activities. Traditionally, various species from this family have been used to cure fever, for example *Quassia amara* from South America [3], *Eurycoma Longifolia* from South East Asia [4] and *Q. africana* Baill. from Africa [5]. These species also are known to have a very bitter substance due to their secondary metabolites which is tryptophan-derived alkaloids and triterpene-derived quassinoids. Some such plant-deprived quassinoids have been shown to possess anti-amoebic [6,7], antifeedant [8], anti-inflammatory[9], antitumour [10] and antiviral [11] properties. Most importantly, quassinoids such as bruceantin, simalikalactone D, glaucarubinone and soularubinone have been shown to be highly active against chloroquine-resistant strains of *P. falciparum in vitro*[12,13]. With the awareness of development of quassinoids as antimalarial drugs, it becomes relevant to evaluate *Quassia* plant. There are two species of *Quassia* L. that can been found in Sabah and Sarawak of Malaysian Borneo, which are *Q. borneensis* Noot and *Q. indica* Linn.

Q. borneensis (Figure 1) is uncommon but can be found in primary mixed dipterocarp forest. Both trees are being used by indigenous people to treat hypertension [14]. Interestingly, previous study [15] had shown that the extracts of *Q. borneensis* possessed antimalarial activity when evaluated using the pLDH assay. In the present study, evaluation was done by using SYBR Green I assay which is a highly sensitive for DNA synthesis indication in order to determine the level of sensitivity of the chloroform extract of *Q. borneensis* towards the different life cycles of parasites. Quassinoids may be presumed to act upon the malaria parasite through a fundamentally different mechanism and so to be further investigated as a potentially useful agent in the treatment of chloroquine-resistant malaria [16].

2.0 EXPERIMENTAL

2.1 Plant Materials

Samples of the plant were acquired from a fully grown tree and identified by Botanist of Forest Research Centre (FRC), Sepilok, Sandakan, Sabah, Malaysia. Its herbarium specimen was prepared and deposited in the Herbarium. Plant's samples were classified to twigs, wood, leave, root and bark before they were processed to powder form and stored in a -15°C chill room. The voucher specimen is deposited under the identification number SAN 152508.

Figure 1 *Q. Borneensis* Noot.

2.2 Extraction

All chemicals used in plant extraction were of analytical grade from Merck (Germany). About 1 kg of powder form plant samples were defatted in petroleum ether and soxhlet in methanol for 2 days before concentrated in vacuo. The concentrated methanol extracts were suspended in 10% aqueous methanol and partitioned between *n*-hexane and chloroform. The pooled extracts of hexane and chloroform were concentrated in vacuo producing hexane and chloroform extracts. The aqueous portions were freeze-dried to acquire aqueous extracts. These hexane, chloroform and aqueous extracts were labelled as *Q. borneensis* soxhlet extracts.

2.3 Experimental Animals

All mice used throughout the study were obtained from Laboratory Animal Resource Unit, UKM Medical Centre, Kuala Lumpur and were housed at the Animal House UKM Kuala Lumpur at a temperature of 25-30°C. Food and beverages provided were rat pellets and water *via* ad libitum. Adult ICR mice strains were also selected from the male mice weighing between 25-30 g.

2.4 Malaria Parasites

Strain of *P. berghei* NK65 were obtained from malaria-infected mice stocks of Biomedical Science Programme, Universiti Kebangsaan Malaysia (UKM).

2.5 Ethical Considerations

Experiments with animals were carried out with the approval from Animal Ethics Committee, Universiti

Kebangsaan Malaysia (FSK/BIOMED/2013/ROHI/25-SEPT./540-SEPT.-2013-MARCH-2015).

2.6 Experimental Procedures

Infected mice with 10% parasitemia were sacrificed and the blood was taken by cardiac puncture. In this study, the level of 10% parasitemia was chosen based on previous study [17] which found out that in the 10% of parasitemia, the presence of mature trophozoite is very high. Blood filtration was done by using cellulose fibrous to get the hematocrit [18]. Parasites were synchronized to a maximum age differential of 6 hours by a sorbitol lysis [19]. To establish synchrony, each culture was centrifuged at 1800 rpm for 5 min, the supernatant discarded, and the pellet resuspended in 2.5 mL of aqueous 5% D-sorbitol for 5 min at room temperature. After an additional centrifugation, an equal volume of RPMI-1640 containing 10% fetal bovine serum was added to the pellet. Every 6 hours, the culture from 6 well plates was transferred into a 96 well microplate and incubated with the chloroform *Q. borneensis* extracts (Q1-Q4) for 24 hours. The *Q. borneensis* extracts were investigated using SYBR Green I – based fluorescence assay [20] with modifications. An aliquot of parasite inoculums (25 μL) with 10% parasitemia and 25% hematocrite was added into each well of microtiter plate.

After 24 hours incubation, 100 μL of lysis buffer [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008% w/v) and Triton X-100 (0.08% v/v)] containing SYBR Green I dye (Invitrogen, United States) was added prior to 1 hour incubation in dark. Extracts of *Q. borneensis* (dissolved in DMSO and diluted with RPMI-1640 to final concentration of 1%) was added to malaria culture at seven final concentrations (0.0001-100 mg/mL). Chloroquine (CQ) and artemisinin (ART) were used as standard antimalarial drugs because both of them have different mechanism of action and were active at different stages of *P. berghei*'s life cycle. The experiment was carried out in triplicate. IC_{50} value was used as an indicator of antimalarial activity and calculated by Graphpad Prism (Graphpad Prism for Windows, version 6.0; Graphpad Software, Inc., San Diego, CA). Values are mean ± SEM for 3 replicates experiment.

3.0 RESULTS AND DISCUSSION

In the present study, four chloroform extracts prepared from bark and root of *Q. borneensis* were screened ex vivo for their antimalarial activities. According to the WHO recommendation and previous works [21,22], antiplasmodial activities of plant extracts were classified as follows: highly active extracts with IC_{50} <5 μg/mL, promising activity at 5-15 μg/mL, moderate activity at 15-50 μg/mL and inactive at >50 μg/mL. In our results, extracts of *Q. borneensis* had been subjected to synchronization test in order to assess their inhibition capability

towards the specific stages in *P. berghei* life cycle (ring, trophozoite & schizont) compared to chloroquine and artemisinin as positive control. The normal and infected erythrocytes are shown in Figure 2.
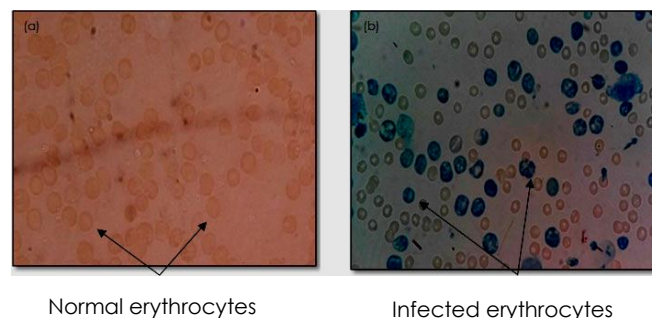

Normal erythrocytes   Infected erythrocytes

Figure 2 (a) Thin blood film of normal erythrocytes, (b) Thin blood film of infected erythrocytes at 10% parasitemia under 1000x magnification by Field's stain

The IC_{50} values obtained from GraphPad Prism software was used as parameter for antiplasmodial activity where it will refer to 50% of concentration of extracts that could inihibit *Plasmodium*. Table 1, shows that all of the extracts had highly active activity of antiplasmodial towards all stages of parasites's life cycles and there was no significant difference (p>0.05) of IC_{50} values compared with the positive controls. Our findings concluded that the potential of *Q. borneensis* as antimalarial agents is comparable with chloroquine and artemisinin.

Our finding is in line too with the previous study [15] which also found that the chloroform extracts of *Q. borneensis* had lowest IC_{50} than hexane and aqueous extracts against different life cycle of *P. berghei* by using pLDH assay. In addition, most of the quassinoids that have potent antimalarial activities are relatively nonpolar compounds and poorly soluble in water [23].

Based on the previous study [24], synthesis of nucleic acids (DNA and RNA) increased slowly through the ring stages, rapidly peaked in late trophozoite and early schizont, but declined as schizogony reached completion. In our study, all of the extracts showed low IC_{50} at the schizont rather than the ring and trophozoite stages. These results agree with previous study [25] that simalikalactone E (SkE), a compound that was isolated from *Q. amara* had better inhibitory effects during schizont stage too. In addition, the *Plasmodium* culture that was treated with Simalikalactone D (SkD) showed that, the IC_{50} level dropped to 10 nM at the 30 hours, when the production of plasmodial DNA is maximal and rate of protein synthesis is still elevated [26]. DNA and protein synthesis inhibition in *P. falciparum* has been reported for several quassinoids and in general but the inhibition of DNA synthesis was less pronounced and seemed to be a consequence of protein sysntheis inhibition [16]. Thus, from our finding, we postulate that *Q. borneensis* has a better inhibitory effect on

stages where DNA synthesis occurred but further study to confirm the mechanism of action of the

active compounds from this plant needs to be done.

Table 1 Ex vivo antiplasmodial activity (IC_{50}) of extracts of Q. borneensis at different stages of life cycle of P. berghei

Extracts	Part	Method	IC_{50} (mg/mL) ($\pm$ SEM)		
			Ring	Matured Trophozoite	Schizont
Q1	Bark	Maceration	4.994×10^{-6} (0.2432)*#	2.705×10^{-6} (0.2806)*#	2.655×10^{-6} (0.2115)*#
Q2	Root		1.291×10^{-6} (0.4406)*#	1.906×10^{-6} (0.4845)*#	1.163×10^{-6} (0.6932)*#
Q3	Bark	Soxhlet	7.481×10^{-6} (0.1815)*#	2.465×10^{-6} (0.4534)*#	1.022×10^{-5} (0.1532)*#
Q4	Root		6.046×10^{-6} (0.4056)*#	2.828×10^{-6} (0.2095)*#	1.00×10^{-5} (0.1411)*#
CQ			1.551×10^{-5} (0.1193)	3.948×10^{-6} (0.2200)	4.124×10^{-6} (0.1922)
ART			1.038×10^{-5} (0.1567)	4.928×10^{-6} (0.1163)	$1.208 \ 10^{-5}$ (0.1161)

Values are mean $\pm$ SEM for 3 replicates experiment
*Significantly difference (p<0.05) with Chloroquine (CQ)
Significantly difference (p<0.05) with Artemisinin (ART)

In contrast, chloroquine and artemisinin showed the lowest IC_{50} at trophozoite stage. These results were supported by the reports [27] which showed that chloroquine and artemisinin acted on early ring stages and will enhanced clearance of parasites shortly after administration and potentially preventing further development of susceptible parasites and worsening of clinical illness [28]. Previous report [16] showed that matured trophozoite and schizont stages were most sensitive to chloroquine since 4 hours exposure to the drug. Meanwhile, artemisinin showed the lowest IC_{50} at the trophozoite stage than others stages. From our experiments, we concluded that active compounds from Q. borneensis have a potential to be developed as an antimalarial agent and schizonticide. There is a also subsequent inhibitory effect upon nucleic acids and may be presumed to act upon the malaria parasite through a fundamental different mechanism, and warrant further investigation as potentially useful agent in the treatment of chloroquine-resistant malaria. Further investigations are also necessary before it can be concluded whether inhibition of protein synthesis or inhibition of other cellular processes underlies the specific mode of action of these active compounds as antimalarial agents.

4.0 CONCLUSION

As a conclusion, the chloroform extracts of roots and barks of Q. borneensis showed potent activities towards each life cycle of chloroquine-resistant P. berghei and have a potential to be developed as an antimalarial agents. Further studies are needed to fully understand the mechanism by which active compounds from the plant that can inhibit the growth of P. berghei.

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