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Bioactive compounds fractionated from endophyte *Streptomyces* SUK 08 with promising *ex-vivo* antimalarial activity

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ABSTRACT

Objective: To determine *ex vivo* antimalarial activity and cytotoxicity of endophytic *Streptomyces* SUK 08 as well as the main core structure fractionated from its crude extract.

Methods: The activities of SUK 08 crude extract were evaluated by using the *Plasmodium* lactate dehydrogenase assay and synchronization test against rodent malaria parasite *Plasmodium berghei*, instead of human malarial parasite *Plasmodium falciparum*. The cytotoxicity of the crude extract was determined by MTT assay. The crude extract was analyzed by thin-layer chromatography and gas chromatography–mass spectrophotometry.

Results: The ethyl acetate crude extract showed very promising antimalarial activity with IC_{50} of 1.25 mg/mL. The synchronization tests showed that ethyl acetate extraction could inhibit all stages of the *Plasmodium* life cycle, but it was most effective at the *Plasmodium* ring stage. On the basis of a MTT assay on Chang Liver cells, ethyl acetate and ethanol demonstrated IC_{50} values of >1.0 mg/mL. The IC_{50} of parasitemia at 5% and 30% for this extract was lower than chloroquine. Thin-layer chromatography, with 1: 9 ratio of ethyl acetate: hexane, was used to isolate several distinct compounds. Based on gas chromatography–mass spectrophotometry analysis, three core structures were identified as cyclohexane, butyl propyl ester, and 2,3-heptanedione. Structurally, these compounds were similar to currently available antimalarial drugs.

Conclusions: The results suggest that compounds isolated from *Streptomyces* SUK 08 are viable antimalarial drug candidates that require further investigations.

1. Introduction

Malaria remains as one of the most devastating vector-borne parasitic diseases in humans [1]. Enhanced by factors such as global warming, climate change, and human migration, about 3.3 billion people live in malaria endemic countries. Moreover, 91% of the yearly deaths reported from the African continent range from 537 000 to 907 000 cases as documented in the 2011 World Malarial Report by the World Health Organization [2]. Nowadays, *Plasmodium falciparum* (*P. falciparum*), the most dangerous human malaria parasite, shows resistance to almost all antimalarial drugs [3]. As a result, a crisis has been created, greatly weakening the therapeutic weapons used to combat the disease and creating an imbalance between effective antimalarial drugs and emergence of resistant *Plasmodium* [4]. Specifically,

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chloroquine resistance is frequently encountered. There is further complication due to recent clinical and laboratory evidence of resistance to other mainstream antimalarial drugs such as sulfadoxine, pyrimethamine, and quinine, all of which also require immediate attention. Recently, emergence of human malaria parasite *Plasmodium knowlesi* was reported in Malaysia [5]. *Plasmodium knowlesi* is assumed to be the fifth species to cause malaria in humans [6].

Streptomyces is a Gram-positive bacterium and the largest genus of the Actinobacteria phylum. Despite the fact that *Streptomyces* produces nearly 80% of antibiotics as well as other classes of biologically active secondary metabolites [7], no in-depth research has been conducted on endophytic microorganisms. Pursuing investigations in identifying key bioactive compounds may reveal many potential therapeutic applications and be valuable to many scientific disciplines and medicine [8]. Particularly, *Streptomyces* bioactive compounds have immense potential to combat malaria. *Streptomyces* NRRL 30562 produces the secondary metabolite known as munumbicin D [9]. Munumbicin D inhibits the growth of *P. falciparum*, with an IC_{50} value of (4.50 ± 0.07) ng/mL, which is much lower than chloroquine. Besides munumbicin D, metabolites such as coronamycin have been identified and shown to possess antimalarial activity. To note, coronamycin is produced by *Streptomyces* sp. MSU-2110 [10].

The structure of *Plasmodium berghei* (*P. berghei*) (a key rodent malaria species) lactate dehydrogenase has been studied extensively as a model to get an insight into *P. falciparum* LDH structure. Winter *et al.* [11] confirmed there is a similarity between *P. falciparum* LDH structure and *P. berghei* lactate dehydrogenase enzyme structures, thus supporting the experimental use of *P. berghei* for testing potential antimalarial activity. This parasite is highly dependent on red blood cells during its glycolytic cycle and catalyzes *Plasmodium* lactate dehydrogenase assay (*p*LDH) to produce ATP as its energy source to ensure survival [12]. Therefore, drugs that can inhibit *p*LDH could lead to the death of the parasite, and such drugs may have great potential as viable antimalarial agents [13].

Streptomyces SUK 08 is an endophytic bacterium isolated from the plant *Scindapsus hederaceus*, also known as 'selusuh sawa' in Malay. According to traditional medical practices, selusuh sawais used to treat pregnant women during labor [14] and to treat arthritis [15]. As documented in a previous study, SUK08 also has very good antibacterial and antifungal activity against *Bacillus subtilis* (54%) and *Aspergillus fumigatus* (100%), respectively [16]. The aim of this study was to assess the antimalarial activity of SUK 08 crude extract by using four types of solvents as well as different *P. berghei* parasitemia levels and parasitic stages. Cytotoxicity effect of SUK 08 extracts on human cell lines was also determined and bioactive compounds from these extracts were identified.

2. Materials and methods

2.1. Cultivation and extraction of Streptomyces SUK 08

The Novel Antibiotics Research Group, Universiti Kebangsaan Malaysia (UKM), provided the *Streptomyces* SUK 08 stock culture. The isolate was grown on International Streptomyces Project-2 Agar and incubated in room temperature (RT) 28–30 °C for 14 days. Then, five blocks of agar (2 mm × 5 mm × 5 mm) of matured *Streptomyces* SUK 08 was added to 600 mL of nutrient broth. The broth was incubated for 21 days at RT with shaking at 200 rpm. Then, it was homogenized and successively extracted using hexane, dichloromethane, ethyl acetate, and ethanol. The solvent phases contained crude extract of secondary metabolites and were dried using a rotary evaporator. For extraction using ethanol, a method developed by Isnansetyo and Kamei [17], with some modifications, was carried out. Briefly, *Streptomyces* SUK 08 plates were incubated at RT for 14 days. Then, *Streptomyces* SUK 08 cultures were dissolved and extracted with 300 mL ethanol. The supernatant of the ethanol extract was then dried and concentrated using a rotary evaporator. All the crude extracts, regardless of solvent used, were diluted with ethyl acetate and Roswell Park Memorial Institute-1640 (RPMI-1640) media until the final concentration was 0.000 1 μg/mL to be used for downstream assays.

2.2. P. berghei infection in mice

Permission to use animals for our studies was approved by the UKM Animal Ethical Committee (UKM/Noraziah/341/Feb2011). Male ICR strain mice weighing 20–30 g and 8–12 weeks in age were obtained from the Experimental Animal Unit, Faculty of Medicine, UKM. These mice were daily feed *ad libitum* with water and pallet. To initiate *P. berghei* infection, 30–40 μL/mL blood was taken from the tail tip of *P. berghei*-infected donor mice and serially diluted with the Alsever's solution to obtain 1×10^6 parasitized red blood cells. Uninfected mice were intravenously administered with 0.2 mL of this suspension to obtain the parasitemia density (%) by thin blood film. At a particular parasitemia density, the mice were then diethyl ether-sacrificed, and infected blood was taken out by cardiac puncture and collected into tubes containing 0.002 5% trypsin–EDTA solution. The blood was filtered using CF11 powdered medium before being centrifuged at 16 000 rpm for 5 min. To this suspension, 15 mL RPMI-1640 was added and centrifuged again until the supernatant became clear. Then, the supernatant was discarded, leaving the RBC pellet (the hematocrit).

2.3. Ex vivo antimalarial test

Crude extracts were assessed for *ex vivo* antimalarial activity using a modified *p*LDH method as described previously [18,19]. Crude extracts were first dissolved in 10% DMSO, vortexed, and then diluted in malaria culture medium to prepare 2 mg/mL solution. Micro-titration technique was used to measure the activity of samples at various concentrations of (2 000.000 0–0.000 1) μg/mL. Five percent hematocrit was aliquoted as 95 μL extracts into 96-well microtiter plates (Nunclon, Denmark) and incubated at 37 °C for 24 h. Chloroquine diphosphate (Sigma-Aldrich, USA) was dissolved in distilled water (2 mg/mL) and served as the control in all experiments. All tests were carried out in triplicate. After incubation, the plates were frozen at −80 °C for an hour and then thawed at RT to lyse red blood cells. At the end of the incubation, the cultures were suspended, and an aliquot for 20 μL was removed and added to 20 μL of Malstat-PES reagent (Sigma-Aldrich, USA) in a 96-well microtiter plate. *p*LDH activity was determined via spectrophotometry. Briefly, 20 μL of Nitro Blue Tetrazolium, and parasite growth was determined as the optical density (OD) at 655 nm