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

Noraziah Mohamad Zin[1*], Juwairiah Remali[1], Mohd Nazir Nasrom[1], Shafariatul Akmar Ishak[1], Mohd Shukri Baba[2], Juriyati Jalil[3]

[1]*School of Diagnostic Sciences and Applied Health, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia*

[2]*Department of Biomedical Science, Kulliyyah of Allied Health Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia*

[3]*Drug and Herbal Research Centre, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*

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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,

*Corresponding author: Noraziah Mohamad Zin, Programme of Biomedical Science, School of Diagnostic Science and Applied Health, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia.
Tel: +60 392897373 (office), +60 193924639 (mobile)
Fax: +60 326923094.
E-mail: noraziah.zin@ukm.edu.my (N.M. Zin).

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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

(OD_{655}). The antimalarial activity of crude extracts was expressed as IC_{50} values. The OD_{655} values from control wells devoid of crude extracts or with the control drug were referred to as having 100% *p*LDH activity.

2.4. Sorbitol synchronization

Synchronization of *P. berghei*-infected erythrocytes was based on the method described in Ref. [20] with some modification. Briefly, parasites were taken when they were mostly at the ring stage, which was no more than 10–12 h post-inoculation when the sorbitol treatment was completely done. Blood hematocrit was mixed with 5 mL of 5% D-sorbitol solution (Sigma-Aldrich, USA), incubated at 37 °C for 10 min, and was shaken 2–3 times right after being centrifuged at 19000 rpm for 10 min. Then, at the same speed and time, the mixture was centrifuged again with malaria culture medium to remove the sorbitol. The supernatants were removed carefully, which contained the knobby parasites, without touching the pellets [21]. Thirty microliters of hematocrit was mixed into 10 mL RPMI-1640 and incubated for 6 h, 16 h, and 22 h. The antiplasmodial effect of SUK 08 crude extract was determined using light microscope (Olympus, Japan) at ×40 magnification. The various life stage; young trophozoite (ring stage), late trophozoite, and schizont was observed at 6 h, 6 h and 22 h. Antimalarial studies using the *p*LDH assay were carried out as described previously.

2.5. Cytotoxicity test

Cytotoxicity of SUK 08 crude extracts was determined by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay [22]. The extract was dissolved in DMSO and diluted with RPMI-1640 to achieve the desired concentration. Chang Liver cell lines (ATCC CCL-13, Manassas, VA 20108 USA) were cultured at 37 °C and 5% CO_2 in RPMI-1640 (Gibco, USA) with 10% fetal bovine serum (Gibco, USA). The concentration of Chang Liver cell suspension was adjusted to 5.0×10^4 cells/mL and was pipetted into each well of 96-well microtiter plates. After being treated with 200 µL crude extract for 24 h, cell viability was determined by the MTT assay. DMSO was used to dissolve formazan crystals, and absorbances of the samples were read at 570 nm using an iMark Microplate Absorbance reader (Bio-Rad, USA) to determine IC_{50} values.

2.6. Thin layer chromatography

The crude extract of *Streptomyces* SUK 08 was dissolved in ethyl acetate. An aliquot of the ethyl acetate-crude extract was spotted on a 9.6 cm × 2.0 cm thin layer silica gel plate. Then, the plate was developed in 1:9 ratio of ethyl acetate: hexane medium. The dried thin-layer chromatography was sprayed with anisaldehyde to visualize color of the bands.

2.7. Gas chromatography–mass spectrophotometry

Using hexane as the mobile phase, the crude extracts were analyzed using GCMS-QP5050A (Shimadzu, Japan) via a 30 mm and 0.32 mm-sized HP-5 column at 150 °C and 280 °C for both the inject and detector ports.

3. Results

3.1. Early antimalarial screening and cytotoxicity

The ethyl acetate-SUK 08 extract (IC_{50} 1.25) had the highest antimalarial activity as compared to chloroquine (IC_{50} > 2) (Table 1). This extract had the lowest IC_{50} without cytotoxicity effects on Chang Liver cells (Table 1). Thus, this extract was further examined for antimalarial activity at low and high parasitemias. Specifically, this extract's antimalarial activity was explored at 5%, 10%, and 30% parasitemia density as based upon the severity of infection in mice.

3.2. Antimalarial screening of ethyl acetate-SUK 08

From the thin blood film at different parasitemia densities, all stages of the *Plasmodium* life cycle were observed. To note, at 5% of parasitemia, there were huge amounts of young trophozoites (rings) with fewer numbers of matured trophozoites and schizonts. At 10% of parasitemia, there were many matured trophozoites with very little schizonts. Lastly, at 30% of parasitemia, almost all of the cells were at the gametocyte stage. Obviously, the IC_{50} of the extract at 5% and 10% was more effective as compared to chloroquine, which was estimated to be more than 2 mg/mL at the same parasitemia level. The IC_{50} value of ethyl acetate at 5%, 10% and 30% parasitemia are 1.583, 1.250 and 1.001 mg/mL, respectively. IC_{50} value of chloroquine at 5%, 10% and 30% parasitemia are >2.00, >2.00 and 1.75 mg/mL, respectively.

3.3. Inhibition of Plasmodium life cycle

Plasmodium stages over 24 h were observed and determined under light microscope. The results showed that young trophozoites were observed at 6 h, while late trophozoites and schizonts were observed at 16 h and 22 h, respectively. It was clear that the SUK 08 crude extract could inhibit all *Plasmodium* stages. The IC_{50} value at young trophozoite (ring), mature trophozoite, schizont and all stage of *Plasmodium* life cycle are 260.96, 770.00, 1125.00 and 737.69 µg/mL, respectively.

3.4. Separation of the SUK 08 crude extracts

From the separation of the crude extract, nine compounds were successfully obtained by using thin-layer chromatography and a 1:9 ratio of ethyl acetate: hexane solvent system.

3.5. Gas chromatography–mass spectrometry analysis

The separation of bioactive compounds from SUK 08 crude extracts were determined by gas chromatography–mass

Table 1
IC_{50} value of early antimalarial screening and cytotoxicity (mg/mL).

Extract	IC_{50} *P. berghei*	IC_{50} Chang liver cells
Ethanol-SUK 08	>2.00	>1.00
Ethyl acetate-SUK 08	1.25	>1.00
Dichloromethane- SUK 08	1.30	0.40
Hexane-SUK 08	>2.00	0.89
Chloroquine	>2.00	[a]

[a] Test is not done.

Table 2
Spectrum obtained in GC–MS data analysis.

Peak	R. Time	I. Time	F. Time	Area	Area%	Height	Height %	A/H	Mark name
1	2.035	2.017	2.167	115025	3.47	31970	1.57	3.60	Hexane
2	2.197	2.167	2.275	406756	12.27	268529	13.20	1.51	Cyclohexane
3	2.298	2.275	2.333	49190	1.48	38903	1.91	1.26	Hexane, 3-methyl-
4	2.417	2.392	2.458	7396	0.22	4331	0.21	1.71	Heptane, 4-azido-
5	2.585	2.550	2.675	1807109	54.50	1287381	63.27	1.40	Heptane
6	2.956	2.917	2.983	23420	0.71	14617	0.72	1.60	Cyclopropane, 1,1,2,2-tetramethyl-
7	3.105	3.050	3.125	7236	0.22	2524	0.12	2.87	Propane, 2-nitro-
8	3.813	3.783	3.833	11376	0.34	6683	0.33	1.70	Oxalic acid, butyl propyl ester
9	4.014	3.983	4.058	17457	0.53	9432	0.46	1.85	Acetyl valeryl
10	4.830	4.775	4.892	870581	26.26	370422	18.20	2.35	Octane
–	–	–	–	3315546	100.00	2034792	100.00	–	–

spectrometry. The analysis managed to identify 10 compounds. Using hexane as the mobile phase, the highest peak manifested at 2.585 min, followed by the next peak at 4.830 min (Table 2). The MS for each peak was compared with those documented in library data for their chemical structure. Butyl propyl ester, cyclohexane, and 2,3-heptane dione compounds were among the most common compounds.

4. Discussion

To determine the best solvent for crude extract extraction of SUK 08 compounds, several types of solvents were tested. Gessler *et al.* [23] highly recommended implementing the extraction method using solvents in increasing order of polarity. Generally, non-polar solvents will selectively dissolve non-polar compounds and vice versa. In this study, hexane was used as a nonpolar solvent, ethyl acetate and dichloromethane as semi-polar solvents, and ethanol as a polar solvent.

Out of these four different solvents, ethyl acetate was the best solvent for crude extract extraction. Ethyl acetate-SUK 08 crude extracts exhibited the highest antimalarial activity against *P. berghei*. Such observations are most likely due to the semi-polar nature of ethyl acetate, thus attracting both polar and nonpolar SUK 08 compounds in the crude extract. The combination of polar and nonpolar compounds provides a synergistic effect not observed in the antimalarial action of chloroquine, which is a pure compound. As documented by Willcox [24], the crude extract with a combination of several soluble compounds were shown to be more active as compared to individual compounds. However, Zin *et al.* [25] stated that under certain conditions, only secondary metabolites that are soluble in ethyl acetate are extracted efficiently. Thus, there may be other bioactive compounds missed due to insolubility in the ethyl acetate solvent.

Dua *et al.* [26] classified extracts as non-toxic to human cell lines if the IC_{50} was greater than 16 mg/mL. Whilst Malebo *et al.* stated extracts as non-toxic if the IC_{50} was greater than 30 mg/mL [27]. Therefore, since all extracts in our study possessed IC_{50} values exceeding 16 g/mL and 30 mg/mL, there were considerably not toxic to these normal human cell lines. Regarding parasite inhibition, the ethyl acetate-SUK 08 crude extract inhibited all stages but best inhibited gametocytes. Since *Plasmodium* can shift its morphology throughout its life cycle, their genetic properties and components could be changed as well [18]. Therefore, it is not surprising if the production of *p*LDH enzyme varies at different parasitic stages [18].

Some antimalarial drugs possess esters such as methyl ester, ethyl ester, propyl ester, and butyl ester as their main functional group. Ester groups were proven to confer antimalarial activity when 5-aminolevulinic acid was demonstrated as a potential antimalarial agent due to the attachment of an ester group as part of its derivative structure [28]. Additionally, the series of *n*-alkyl or aryl ester groups have great potential as *in vitro* antimalarial drugs [29]. The newly discovered antimalarial drug fosmidomycin contains derivatives of an acyloxy alkyl ester and is able to inhibit the biosynthesis of the malaria isoprenoid enzyme [30]. There are similarities between 5-aminolevulinic acid with butyl propyl ester and 2,3-heptanedione compounds fractionated from SUK 08 crude extracts. To note, butyl propyl ester has similarity to characterized 5-aminolevulinic acid derivatives. $CH_3HC_2CH_2$-*n* is a derivative structure of propyl, and $CH_2CH_2CH_2CH_3$ is a derivative structure of butyl. The functional group of ester is COOR, where R is attached to any alkyl group. The 2,3-heptanedione also possessed the same derivative structures, which was an addition of a methyl group ($COOCH_3$). However, the role of ester functional groups is not only crucial to antimalarial activity, but these groups in quassinoids compounds have antiviral properties [31].

Most antimalarial drugs are found in cyclo-derivative form [32–34]. However, the derivative of cyclohexane is in the form of a weak base [35]. *In vitro*, cyclohexane, an analog to antimalarial drug arterolane, was less sensitive to *P. falciparum* yet exhibited potent activity against *P. berghei* in infected mice [35]. Solely administered as a pure compound, cyclohexane was not active against malaria [36].

Although the 16S rRNA partial sequence and BLASTN result of two independent *Streptomyces* SUK 08 samples studied by Sarmin *et al.* [16] showed 99% similarity to *Streptomyces eurythermus*, our study found that the ethyl acetate-crude extract of endophytic *Streptomyces* SUK 08 showed a novel antimalarial activity without any significant cytotoxicity effect on Chang Liver cells. While it is almost impossible to produce a vaccine capable of inhibiting the parasite life cycle, this crude extract could inhibit all stages of the life cycle, inhibiting the gametocyte stage the most. Moreover, the SUK 08 crude extract inhibited the multiple parasitemia levels. With these promising results, there is a vital need to explore the actual chemical structure of the compounds found in the extract and identify mechanisms of antimalarial action by these compounds. Such mechanisms of action may be novel; however, we do note that the functional derivatives or active groups of the compounds we discovered are similar to several currently available antimalarial drugs.

Conflict of interest statement

We declare no conflicts of interest.

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