In-vivo antimalarial activity of the endophytic actinobacteria, *Streptomyces* SUK 10

Mohd Shukri Baba[1], Zainal Abidin Abu Hassan[2], Jalifah Latip[3], Florence Pethick[4], Iain S. Hunter[4], RuAngelie Edrada-Ebel[4], Paul R. Herron[4] and Noraziah Mohamad Zin[1*]

[1] School of Diagnostic Sciences and Applied Health, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia.

[2] Department of Parasitology, Medical Faculty, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.

[3] School of Chemistry Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Malaysia.

[4] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, United Kingdom.

* Corresponding author:
Telephone: +60392897373(office), +60193924639 (mobile)
Fax: +60326923094
Email: noraziah.zin@ukm.edu.my

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ABSTRACT

Endophytic bacteria, such as *Streptomyces*, have the potential to act as a source for novel bioactive molecules with medicinal properties. The present study was aimed at assessing the antimalarial activity of crude extract isolated from various strains of actinobacteria living endophytically in some Malaysian medicinal plants. Using the four day suppression test method on male ICR strain mice, compounds produced from three strains of *Streptomyces* (SUK8, SUK10 and SUK27) were tested *in vivo* against *Plasmodium berghei* PZZ1/100 in an antimalarial screen using crude extracts at four different concentrations. One of these extracts, isolated from *Streptomyces* SUK10 obtained from the bark of *Shorea ovalis* tree, showed inhibition of the test organism and was further tested against *P. berghei*-infected mice for antimalarial activity at different concentrations. There was a positive relationship between the survival of the infected mouse group treated with 50 µg kg^{-1} body weight (bw) of ethyl acetate-SUK10 crude extract and the ability to inhibit the parasites growth. The parasite inhibition percentage for this group showed that 50% of the mice survived for more than 90 days after infection with the parasite. The nucleotide sequence and phylogenetic tree suggested that *Streptomyces* SUK10 may constitute a new species within the *Streptomyces* genus. As part of the drug discovery process, these promising finding may contribute to the medicinal and pharmaceutical field for malarial treatment.

INTRODUCTION

Climate change and population movement are among the factors that have contributed to an increase in disease (Pillay and Zambon, 1998; Espinel *et al*., 2001). Malaria is the most significant human parasitic disease that increasingly threatens many of the world's

population, particularly children and the elderly due to the emergence of drug resistance (Joy *et al*., 2003; Bray *et al.*, 2005; Yotoko and Elisei, 2006). Chloroquine resistance is common in Malaysia and most parts of South East Asia (Wellems, 2002; Abdulelah and Zainal-Abidin, 2007) and there is also clinical and laboratory evidence of resistance to sulfadoxine or pyrimethamine in combination as well as quinine (Wellems, 2002).

Tropical forests in Malaysia are among the oldest on the planet and as many as 1,300 plants from this environment are claimed to have medicinal properties. However, most of them have not been scientifically studied (Ibrahim *et al.*, 1994). Previous findings have showed that the plants used by the native population in Malaysia as alternative medicines provide a habitat for endophytic microorganisms (Behal 2000; Strobel and Daisy, 2003; Zin *et al.*, 2011) and may have potential to treat malaria. Actinomycetes in general, and streptomycetes in particular, produce over two-thirds of clinically useful antibiotics of natural origin (Kurosawa *et al.*, 2000; Berdy 2005; Fiedler *et al*., 2005). These Gram positive bacteria with high G-C content, complex morphological (Flärdh and Buttner, 2009) and physiological differentiation (Bibb 2005). Various reports showed that endophytic members of the genus *Streptomyces* have the ability to inhibit a variety of human pathogens, including bacteria and fungi (Rajendra *et al.*, 2004; Ghadin *et al.*, 2008; Zin *et al.*, 2010; Zin *et al*,. 2011). For example, the endophytic strain *Streptomyces* NRRL 30562 from *Kennedia nigriscans* produces at least one broad spectrum antibiotic (Coombs and Franco, 2003).

In this study, three endophytic *Streptomyces* isolates named SUK8, SUK10 and SUK27 were obtained from the Malaysian plants *Scindapsus hederaceus*, *Shorea ovalis* and *Zingiber spectabile*, respectively. The aim of the present study is to assess the *in vivo* antimalarial activity against *Plasmodium berghei* PZZ1/100 strain by administrating crude extracts of cultures of each endophytic bacterium.

MATERIALS AND METHODS

Plant selection and sterilization technique

Plant voucher number, HM449820 for *S. hederaceus*, HM449822 (*S. ovalis*) and GU238266 (*Z. spectabile*) were submitted to the Universiti Kebangsaan Malaysia (UKM) Herbarium Unit to obtain a tagging code. The sterilization and isolation of the bacteria from the plants were modified from Coombs and Franco (2003). Briefly, the plants were dried at room temperature for 48 hours before being thoroughly washed with tap water. Internal bark of *S. hederaceus* and *S. ovalis* and the external stem of *Z. spectabile* were cut into 5.0 cm long strips and washed with sterile water to remove soil particles and epiphytic microorganisms. The selected part of the plants were then excised and subjected to a three-step procedure of surface sterilization: one minute wash in 99 % (v/v) ethanol, followed by a six minute wash in 3.5 % NaClO (v/v) and a final three-times rinse with sterilized distilled water (sdH_2O). The effectiveness of surface sterilization procedure was tested by plating 1.0 ml of the final rinsed sdH_2O on to nutrient agar. After 37°C incubation for seven days, the sterilization procedure was assumed to be successful if there were no microbial growth on the agar.

Isolation and identification of the bacteria

The outer most skin of all sterilized bark and stem of the plants were cut out. These parts were then aseptically cut as cross-sections into two to three mm thin slices to allow the outer-most, middle and inner-most parts of the samples to be placed on three different agars: actinomycete Isolation Agar (AIA), Starch Yeast Casein Agar (SYCA) and Water Agar (WA) (Castillo *et al.*, 2002). All agars were supplemented with 5.0 ml of 50 µg mL^{-1}

cycloheximide and 50 µg ml^{-1} nystatin as antifungal agents. Bacterial growth on the agar was assessed after four weeks incubation at room temperature (RT) and identified as streptomycetes on the basis of their colony morphology and colour before they were subcultured onto International *Streptomyces* Project 2 (ISP2) agar for the next phase of study.

The endophytic *Streptomyces* colonies were then macroscopically checked using normal light microscope under 40× magnification. Aerial and substrate mycelium colour, dissolved pigment colour and spore chain morphology were among the macroscopic characteristics observed. Gram staining was also done to permit the visualisation of the organism's micromorphology. All of the isolates that displayed the morphological characteristics of *Streptomyces* were then given their UKM's deposition number before they were kept as pure stock culture at -80°C by adding five pieces of (5 × 5) mm thin slices of isolate mycelium to 1.0 ml of glycerol 80% (v/v) (Castillo *et al.*, 2002).

Production of crude extract

Nutrient broth (Sigma-Aldrich, Malaysia) was prepared at different pH levels: 6.5, 7.0 and 7.5. To produce extracts of the isolates, five blocks of 5 × 5 mm ISP2 agar enriched with *Streptomyces* were cut and inoculated into 400 ml autoclaved nutrient broth in a 1000 ml conical flask individually. Fermentations were carried out using an orbital shaker at 28^{o}C at three different rotation rates; 180, 200 and 220 RPM for 19 – 21 days. The supernatant was then extracted three times using the selected solvents at a ratio of 1:2 of pool supernatant. Prior to this, 1200 mL of supernatant were extracted with 200 mL of ethyl acetate three times (Castillo *et al.*, 2002).

***In vivo* antimalarial screening**

All animal experiments were conducted following approval by the UKM Animal Ethics Committee (UKMAEC) coded FSK/Biomed/341. Using ICR strain male mice at 28 – 30 g body weight (bw) and aged six weeks old as the model, the parasite *Plasmodium berghei* PZZ1/100 was administered into the host via the intraperitoneal (IP) route. Every group of mice (n = 6) was housed in a stainless steel cage, treated at room temperature with daily *ad-libitum* feeds at 12-12 hours both with and without a light period. In order to assess the extract activity, the four days (4D) suppression test method (Abdulelah and Zainal-Abidin, 2007) was applied. For the treatment, ethyl acetate-SUK8, -SUK10 and -SUK27 crude extracts were diluted with 1:10 (DMSO:dH_2O). As demonstrated by previous studies (Ata-ur-Rehman *et al*., 1985, Peters and Robinson, 1992, Anthony *et al*., 2005, Abdulelah and Zainal-Abidin, 2007), no physical side effects observed on the treated host with 10% of DMSO. For the positive and negative controls, 10 mg kg^{-1} bw of dH_2O diluted quinine hydrochloride and 0.9% normal saline respectively were used (Peters and Robinson 1992, Abdulelah and Zainal-Abidin, 2007).

Infected blood collected from the tip of the donor mice's tail was serially diluted with Alsever's solution to obtain 1.0 x 10^6 parasitized red blood cells (RBC) before they were IP administered at 0.1 mL to all the tested mice (Abdulelah and Zainal-Abidin, 2007). Within two hours, these mice were immediately treated with 0.1 mL of ethyl acetate-crude extract and also 0.1 mL of both positive and negative control solutions. This point was considered as day zero (D0). Without repeating the parasite infection, the same treatment was repeated for the next three days and referred to as D1, D2 and D3. On D4, a blood smear from the treated mice was stained with Giemsa to obtain parasitemia density (%) and parasitemia inhibition (%). The mice were also observed daily for survival time (day) calculated after 20 days post-infection. Considering that the treatment used in this study was a crude extract and, as

documented in previous studies, (Ata-ur-Rehman *et al*., 1985, Anthony *et al*., 2005, Abdulelah and Zainal-Abidin, 2007), results with a parasitemia inhibition of > 65% were considered as having antimalarial activity and the mouse group with the longest survival time considered as receiving the best treatment. The value for parasitemia density directly reflects the value of inhibition percentage, where the higher the inhibition percentage, the better the treatment effect was (Abdulelah and Zainal-Abidin, 2007), where;

$$\text{Inhibition (\%)} = \frac{\text{Parasitemia of negative control} - \text{Parasitemia of treatment}}{\text{Parasitemia of negative control}} \times 100$$

Treatment for tested and control groups by *in vivo* screening underwent the same procedures with five different narrower concentrations of the extracts; 5, 10, 50, 100 and 200 µg kg^{-1} bw which was implemented only for the best crude extract isolate performed in preliminary screening. Similarly, the best among these five concentrations was determined when the parasitemia inhibition was > 65% in conjunction with the mouse group that showed the best survival.

DNA extraction of endophytic *Streptomyces* isolates

The genomic DNA was isolated by neutral lysis, followed by phenol-chloroform extraction and precipitation with isopropanol. The DNA from the isolates was extracted using a modified protocol (Keiser *et al.*, 2000) where 30 – 50 mg of *Streptomyces* mycelium was suspended with 500 µL lysozyme buffer in a 1.5 mL Eppendorf tube by vortexing before 25 µL of 50 mg mL^{-1} lysozyme and 3.0 µL of 10 mg mL^{-1} RNase were added and the sample vortexed for 10 seconds. At the end of the extraction process, a Thermo Scientific Nano-Drop

2000C machine was then used to determine the purity and concentration reading for the isolated DNA.

Amplification and sequencing of the 23S rDNA gene

Polymerase Chain Reaction (PCR) was performed using the 23S rDNA gene primer pair (P1-5'ACCAGGATGCTTAGAAG3' corresponding to *E. coli* 1051 – 1071 nucleotide sequence of 23S rDNA gene and also P2-5'CACTTACCCCGACAAGGAAT3' corresponding to *E. coli* 1957 – 1938 nucleotide sequence of 23S rDNA gene). PCRs were carried out in 50 µL reactions containing 10 µL *MyTaq* buffer (5× PCR buffer contained 10 mM dNTPs and 25 mM $MgCl_2$), 1.0 µL of both 20 mM reverse and forward primer, 2.0 µL of 5 U/uL *MyTaq* polymerase enzyme. Then 2.0 µL of 60.5 ng/µL DNA template and 34 µL deionized H_2O. The PCR thermocycling conditions were as follows: initial denaturation of DNA template at 95°C for two minutes; followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 54°C for 30 seconds, extension at 72°C for 60 seconds and a final extension at 72°C for five minutes. The resulting PCR products were purified by using the Wizard PCR Prep DNA purification kit (Promega UK) before being sent for sequencing at Eurofins UK using primers P1 and P2. The 23S rDNA sequences obtained were then compared to the Genbank databases by using basic local alignment search tool (BLAST).

Identification of compounds

With hexane and ethyl acetate as the mobile phase A and B respectively, 2.8 g of selected isolate crude extract was fractionated (GRACE Biotage Flash Chromatography). The extract was chromatographed over 40 g generic silica while the columns were detected under 245

and 310 nm of respective UV1 and UV2 absorptions wavelength and coupled with isopropanol as electron light scattering detector (ELSD) at 5.0 mV. The elution was done at 35 mL/min for 80.0 minutes run length. The thin layer chromatography (TLC) analysis was done on all 22 fractions by dissolving the sample with appropriate solvents and was visualized using the UV light with short and long wavelengths of 254 nm and 350 nm respectively. These fractions were selected in order to determine the optimized solvent system that lead to the visualization of the compounds observed in TLC. All of the samples obtained during the fractionation process were dissolved in denatured chloroform ($CDCl_3$) and tested by NMR for determination of its chemical structure and configuration. This method was implemented using NMR AS400 for 400 MHz and Bruker FT-NMR for 600 MHz. The fractions that underwent NMR experiments were subjected for 1D and 2D ^{1}H analysis. COSY (400 Mz), HMBC (6 hours) and HMQC (8 hours) were also further implemented for interesting fractions with promising characteristics (fractions 11, 15 and 20), as well as for ^{13}C and DEPT (both 4 hours) individually. In addition to the NMR profiles, high resonance liquid chromatography mass spectrometry (HR-LCMS) analysis (ACCELA Auto-Sampler, UK) was also carried out and the data were then compared with the information documented in AntiBase library to obtain any supportive data for determination of the compound identity.

Statistical analysis

All results were expressed as the mean ± standard deviation (sd) from six mice per group. Due to the small sample size for each treated group of mice (n = 6), the Shapiro-Wilk test was used to analyse the significance of the antimalarial tests. Statistical significance was

declared when P value was equal to or less than 0.05 ($P \leq 0.05$). Since all groups were normally distributed, no non-parametric tests were required in this study.

RESULTS

Identification of the bacterial strains

Micro- and macro-morphology differences between all three strains were used as a preliminary means to identify the isolates as members of the genus *Streptomyces*. The relation between names, part of the plants, UKM's tagged voucher number, the aerial and substrate mycelium colour, dissolved pigment colour and spore chain morphology, as well as Gram staining colour for all of the tested strains are shown in Table 1. Other than living endophytically in the same part of their respective plants, SUK8 and SUK10 also showed similarities in most of macroscopic and microscopic observations, except for their substrate mycelium colour.

Production of crude extract

After culture optimization, all strains produced most biomass after 21 days fermentation in nutrient broth at pH 7.0 and at an agitation rate of 200 RPM. Extract yields of more than 40 mg were only obtained from all strains with an agitation rate of 200 RPM and nutrient broth at pH 7.0. Consequently these conditions were used for 21 days to isolate SUK10 crude extracts with ethyl acetate for implementing *in vivo* antimalarial screening.

To produce large amounts of crude extract, a scaled-up fermentation with SUK10, the strain that exhibited the strongest activity against *P. berghei* PZZ1/100 was then carried out

using the best variant of pH, aeration rate and optimized length of fermentation. These conditions were characterized based on the highest amount of crude extract produced. This process was repeated in a 1000 mL conical flask using the orbital shaker until the crude extract reached sufficient weight which at least 25.0 mg for *in vivo* antimalarial screening. At the end of the fermentation period, this culture was filtered to separate the mycelium from its supernatant and extracted using ethyl acetate three times before the organic solvent phase was pooled prior to a drying process using a rotary evaporator.

In-vivo antimalarial screening of tested isolates

In order to determine the isolate that exhibited the best antimalarial activities we used three main parameters; parasitemia density, inhibition rate and survival time. For parasitemia density, the SUK10 crude extract at all concentrations (50, 100, 200 and 400 µg kg^{-1} bw) exhibited the lowest value compared with SUK8 and SUK27 (Table 2). With the lowest parasitemia density at 1.08 ± 0.9%, there was a significant difference for this parameter ($P < 0.05$, $n = 6$) at 100 µg kg^{-1} bw between SUK10 and the other two isolates. The same results were also observed for the other three concentrations levels. Meanwhile at 400 µg kg^{-1} bw, 2.64 ± 3.7% was recorded as the highest value of parasitemia density of SUK10; however this was still lower compared with SUK8 (2.98 ± 6.1%) and SUK27 (4.04 ± 5.3%).

For the inhibition rate shown in (Fig. 1A), at all concentrations the SUK10 crude extract exhibited the highest percentage compared with SUK8 and SUK27. With the highest inhibition rate 84.14 ± 2.6% at 100 µg kg^{-1} bw, there was a significant difference ($P < 0.05$, $n = 6$) between SUK10 and the other two isolates at this concentration. As the inhibition rate at more than 65% was considered as a benchmark of *in vivo* antimalarial activity (Ata-ur-Rehman *et al*., 1985, Abdulelah and Zainal-Abidin, 2007), all the percentages observed for

SUK10 were higher than 70%, except 51.44 ± 0.9 % at 400 µg kg^{-1} bw. No inhibition rates higher than 65% were observed for the other two isolates at all concentrations except 71.14 ± 1.2% at 50 µg kg^{-1} bw and 67.45 ± 3.4% at 200µg kg^{-1} bw, both exhibited by SUK8 isolate. Despite this, these values were still lower than those observed from the crude extract of SUK10 isolate at the same concentrations.

It was predicted that survival times of the treated mice would be longer at higher inhibition rates. Mice groups treated with the SUK10 crude extract at all concentrations exhibited the longest survival time compared with the other isolates at the same concentration. In accordance with the inhibition rate, the mouse group treated with SUK10 crude extract at 100 µg kg^{-1} bw managed to survive up to 21 days post-infection as well as 18 and 16 days for the other concentrations (Fig. 1B). There was also a significant difference for the mice survival time ($P < 0.05$, $n = 6$) at 100 µg kg^{-1} bw between SUK10 and the other two strains.

***In-vivo* antimalarial screening of SUK10**

The SUK10 extract was subjected to final *in vivo* antimalarial screening with five different concentrations across a narrower range (5, 10, 50, 100 and 200 µg kg^{-1} bw) in order to determine which concentration gives the best *in vivo* antimalarial activity. With only 1.64 ± 3.0% at 100 µg kg^{-1} bw, there was a significant difference for parasitemia density ($P < 0.05$, $n = 6$) between 100 µg kg^{-1} bw and the other four concentrations. Nevertheless, 1.86 ± 3.1% observed for 50 µg kg^{-1} bw was still lower compared to the other concentrations (Table 3). The blood slides taken on D4 for every concentration of SUK10 treatments from particular mice showed that the distribution of four main stages of *P. berghei* lifecycle were successfully captured in the infected-RBC (Fig. 2). This figure indicated that the complete

development of one plasmodial's lifecycle in suppressed vertebrate host was completed within four days after infection.

Since every concentration had its own negative control group individually, the inhibition rate for 50 µg kg^{-1} bw was relatively high and gave the greatest value, although the parasitemia density was quite lower at 100 µg kg^{-1} bw compared with 50 µg kg^{-1} bw (Fig. 3A). The concentration at these two values, together with the concentration at 200 µg kg^{-1} bw, were considered as the concentrations where antimalarial activity could be best assessed because the value obtained at 5 µg kg^{-1} bw and 10 µg kg^{-1} bw was lower than 65% (Ata-ur-Rehman *et al*., 2005, Abdulelah and Zainal-Abidin, 2007). At the highest value, 70.89 ± 3.1%, there was a significant difference ($P < 0.05$, $n = 6$) between 50 µg kg^{-1} bw and the other four concentrations.

In this study, the longest survival time observed was 91.11 ± 0.5 days post-infection (data not shown), which was exhibited by half of the total mice (n = 3) treated with 50 µg kg^{-1} bw ethyl acetate-SUK10 crude extract. Surprisingly, the remaining half of these mice (n = 3) in this group also managed to survive between 55 – 65 days post-infection which was far longer than the other four groups for SUK10 at this phase (Fig. 3B).

Preliminary identification of SUK10 by 23S rDNA sequencing

Since SUK10 showed the most significant antimalarial activity, the amplification of 23S rDNA gene was only done on this strain. SUK10 samples generated an amplicon close to the predicted size of 1Kb (data not shown). After sequencing, this product was confirmed to be 1006 bp in length with the score 97% of multiple sequence alignments. The BLASTN results of two independent SUK10 sequences revealed that the nucleotide sequence has 94% - 96% identity with other species, with *Streptomyces hygroscopicus jinggangensis* having the

highest homology. The neighbour joining phylogenetic tree (Fig. 4) suggested that *Streptomyces* SUK10 may constitute a new species within the *Streptomyces* genus. Due to this promising outcome, detailed characterization of SUK10 strain is currently being done in our laboratory to determine the full genome sequence of this strain.

Identification of compounds

Twenty-two fractions (V1 – V22) were obtained following separation of spots by TLC (fraction 1 – 7 under development in 80:20 (v/v) of hexane/ethyl acetate, fraction 8 – 13 (60:40, v/v, hexane/ethyl acetate) and fraction 14 – 22 (90:10, v/v, DCM/methanol). The obtained yield and the compound identity name from all fractions of SUK10 crude extract are shown in Table 4. Due to the interesting peak displayed by fraction 15, this fraction was also subjected for ^{13}C and DEPT (600 Mz, 4 hours). The pure fraction in vial number 15 (V15) was revealed as a type of prolyl-leucyl-diketopiperazine namely Gancidin W (Bin *et al*., 2009) based on 1D ^{1}H-^{13}C and 2D ^{1}H-^{1}H NMR analysis, COSY, HMBC, HMQC and DEPT tests of NMR correlation data and Antibase Library data. 16.2 mg of this compound was isolated as a light brownish solid with molecular formula (MF) $C_{11}H_{18}N_2O_2$ and molecular weight (MW) 210.275 at retention time 7.17 minutes. The assignment of the ^{1}H and ^{13}C NMR signal of Gancidin W are shown in Table 5.

DISCUSSION

While the efficacy of the extracts of SUK8 and SUK27 were comparable for all parameters in this study, some of the parameters observed for SUK8 such as parasitemia density at 400 µg kg^{-1} bw and inhibition rate and survival time at 50 µg kg^{-1} bw were approximately the same

value as obtained for SUK10. It was noted that, for all parameters obtained in the final antimalarial screening for SUK10, survival time for the mouse group treated with 100 µg kg^{-1} bw was similar to those treated with 200 µg kg^{-1} bw. The reasons behind this may be multifactorial. Aside from the fact that the progression of the erythrocytic cell cycle in *Plasmodium*-infected host is poorly understood (Prudhomme *et al.*, 2008), every secondary metabolite classes contained in the crude extracts has their own characteristics such as active groups and polarity indexes (Zin *et al.*, 2011) and also, there may be a tendency to obtain more significant values for those parameters in particular cycles or stages of the *Plasmodium* life cycle. These factors were not considered in this study.

Clinically, it is more practical to give the daily treatment with antimalarial drugs containing particular active functional groups for more than seven days (Rajendra *et al.*, 2004; Abdulelah and Zainal-Abidin, 2007), depending on the treatment regime combination, stage of infection and type of plasmodial species (Ernest, 1995; Foley and Tilley, 1998; Kurosawa *et al.*, 2000; Rajendra *et al.*, 2004; Abdulelah and Zainal-Abidin, 2007). At the present time, the chemical structure and active functional groups in all tested crude extracts that are responsible for antimalarial activity has not yet been determined to a high level of detail.

Considering all concentrations both in preliminary antimalarial screening and antimalarial screening of SUK10 extracts, all the untreated control mice died between seven and nine days post infection and more than 18 months post infection for positive control. This was also described in previous *in vivo* antimalarial studies (Zainal-Abidin *et al.*, 1985; Kurosawa *et al.*, 2000; Isaka *et al.*, 2002; Jochen *et al.*, 2002; Abdulelah and Zainal-Abidin, 2007; Prudhomme *et al.*, 2008).

In agreement with the *in vitro* data as well as that observed in other studies (Kurosawa *et al.*, 2000; Isaka *et al.*, 2002; Abdulelah and Zainal-Abidin, 2007; Prudhomme *et al.*, 2008),

it is possible that survival time of the treated mice might be longer at the higher inhibition rate value, which was also observed in this study. Several previous studies (Zainal-Abidin *et al.*, 1985; Kurosawa *et al.*, 2000; Jochen *et al.*, 2002; Rajendra *et al.*, 2004, Abdulelah and Zainal-Abidin, 2007; Prudhomme *et al.*, 2008) found that 50 – 150 µg kg^{-1} bw is the best range of dose for the treatment of crude extract in order to fulfil the same parameters. This may explain why in this study, the parasitemia density rose up at 200 µg kg^{-1} bw (Table 3) before stabilizing at 100 µg kg^{-1} bw in antimalarial screening for SUK10 strain and the inhibition rate decreased at the same concentration (Fig. 3A). To support these findings, some previous studies documented that SUK10 also exhibited very strong antimicrobial activity against *Bacillus subtilis* ATCC 6633 at inhibition percentage 76.2% and *B. cereus* ATCC 6464 (76.4%) as well as antifungal activity against *Aspergillus fumigatus* (84.6%), *Rhizoctonia solani* (94.1%) *Geotrichum candidum* (91.7%), *Trichoderma viridae* and *Fusarium solani* (both 100%) (Ghadin *et al*., 2008, Zin *et al*., 2010).

Based on the ^{1}H NMR analyses in this study, fraction V15 was found to contain interesting signals that corresponded to the major constituent. V15 at 98% purity was isolated at 16.2 mg (0.31% yield) as light brownish solid material with a molecular weight 210.275 and molecular formula of $C_{11}H_{18}N_2O_2$ established by HR-LCMS at a retention time of 7.17 minutes. In this paper, the nomenclature Gancidin W (GW) was assigned for the isolated compound V15. The peak displayed in fraction was determined to be GW. This is a natural compound of the alkaloid group (Adamczeski *et al*., 1995; Bin *et al*., 2009) and as such this compound is suspected to be responsible for antimalarial activity shown in this study. Natural compounds in the alkaloid group are among the most investigated compounds in terms of antimalarial research (Fernandez et al. (2009). For instance, by using the 4D suppressive test on the mice, Cassiarin A that was isolated from the leaves of *Cassia siamea* (Leguminosae) showed an inhibitory effects against *P. falciparum* with IC_{50} value of 0.02 µM (Ekasari et al.

2009). Other than that, alkaloid components isolated from the Bhutanese medicinal plant *Corydalis calliantha* (Fumariaceae) were used for the treatment of malaria in Bhutan. Protopine and Cheilanthifoline showed promising *in vitro* antimalarial activities against *P. falciparum* (both wild type (TM4) and multidrug-resistant (K1) strains, with IC_{50} values of 2.78 – 4.29 μM) (Wangchuk et al. 2010). The ^{1}H and ^{13}C data for V15 (Table 5) were in accordance to the cyclodipeptide structure for *S*-prolyl-*R*-leucyl-diketopiperazine, previously isolated from the sponges *Calyx cf. podatypa* (Adamczeski *et al*., 1995) and *Callyspongia* (Bin *et al*., 2009). A similar compound, named as Gancidin W and elucidated as cyclo-(L-leu-L-pro) but with unknown absolute stereochemistry, was earlier isolated from strains of *Streptomyces gancidus* (Jain *et al*., 1977). In this study, the natural product gave an optical rotation of $[\alpha]_D$ –138.2 (*c* = 1.0, EtOH) which was compatible with those isolated earlier from other *Streptomyces* strains (Jain *et al*., 1977). This diketopiperazine (DKP) derivative compound is naturally produced by many organisms and displays a wide diversity of structures and biological functions (Smaoui *et al*., 2012). This scenario suggests that Gancidin W may be a useful chemical entity for the discovery and development of new drugs. Some DKP derivatives have already been demonstrated to have a good biological activity such as antibacterial, antiviral, antitumor, fungicidal and many more (Belin et al., 2012; Magyar *et al.*, 1999). Although Gancidin W was previously identified in extracts from other *Streptomyces* sp. (Ben Ameur Mehdi *et al*., 2004; Rhee, 2002), its antimalarial properties have not yet been reported.

Consequently through the significance level of statistical tests, the consistency of the values for malarial inhibition in this study are promising and the results reported in this paper indicate that *Streptomyces* SUK10 has good potential as producing an antimalarial agent.

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568 **Table 1.** The respective relationship between plants, its tagged voucher number, macroscopic
569 and microscopic observation of all strains.
570

Strain	Plant	Plant part	Voucher number	Aerial mycelium colour	Substrate mycelium colour	Dissolved pigment colour	Spore chain morphology	Gram stain colour
SUK8	*Scindapsus hederaceus*	Bark (internal)	HM449820	Whitish grey	Light brownish	Yellow	Spiral	Purple
SUK10	*Shorea ovalis*	Bark (internal)	HM449822	Whitish grey	Brownish yellow	Yellow	Spiral	Purple
SUK27	*Zingiber spectabile*	Stem (external)	GU238266	White	Brownish yellow	Yellow	Rectus	Purple

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Table 2. Parasitemia density (%) on day-4 (D4) for treatment and control groups at four different concentrations in preliminary antimalarial screening

Group	Description	Parasitemia density (%)			
		50#	100#	200#	400#
Control	Positive	0.00 ± 0.0*	0.00 ± 0.0*	0.00 ± 0.0*	0.00 ± 0.0*
	Negative	5.03 ± 2.1*	6.82 ± 1.6*	5.17 ± 3.0*	5.44 ± 2.4*
Treatment	SUK8	1.45 ± 0.7*	2.35 ± 4.0*	2.22 ± 0.1*	2.98 ± 3.1*
	SUK10	1.32 ± 1.5*	1.08 ± 0.9*§	1.11 ± 2.7*§	2.64 ± 3.7*
	SUK27	3.11 ± 0.8*	2.23 ± 1.0*	3.33 ± 0.3*	4.04 ± 2.3*

* : Mean ± standard deviation (s.d.)

: Concentration (μg kg^{-1})

§ : $p < 0.05$

Table 3. Parasitemia density (%) on day-4 (D4) for ethyl acetate-SUK10 crude extract treatment and control groups at five different concentrations in antimalarial screening of SUK10 isolate.

Group	Parasitemia density (%)				
	5#	10#	50#	100#	200#
Positive control	0.00 ± 0.0*	0.00 ± 0.0*	0.00 ± 0.0*	0.00 ± 0.0*	0.00 ± 0.0*
Negative control	5.11 ± 2.1*	6.21 ± 1.6*	6.39 ± 3.2*	5.40 ± 2.4*	6.11 ± 0.7*
Treatment	2.32 ± 1.2*	2.18 ± 0.5*	1.86 ± 3.1*	1.64 ± 3.0*§	2.01 ± 0.5*

* : Mean ± standard deviation (s.d.)

: Concentration (µg kg^{-1})

§ : $p < 0.05$

631 **Table 4.** The yield obtained from all isolated fractions of SUK10 crude extract and their

632 compound identity

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Solvent system	Fraction	Net weight (g)	Compound
Hexane and ethyl acetate (H:EA = 8:2)	V1	0.1106	Long chained triglyceride fatty acid
	V2	0.0271	Long chained triglyceride fatty acid
	V3	0.8132	Long chained triglyceride fatty acid
	V4	0.1255	Long chained triglyceride fatty acid
	V5	0.0890	Long chained triglyceride fatty acid
	V6	0.0393	Macrotetrolide B ($C_{47}H_{72}O_{12}$)
	V7	0.0162	Swalpamycin B ($C_{36}H_{58}O_{13}$)
Hexane and ethyl acetate (H:EA = 6:4)	V8	0.0185	Milbemycin A8 ($C_{40}H_{60}O_{10}$)
	V9	0.0125	Phenalamide C ($C_{32}H_{45}NO_4$)
	V10	0.0301	Eicosanedioic acid ($C_{20}H_{38}O_4$)
	V11	0.0249	3-indole lactic acid ($C_{11}H_{11}NO_3$)
	V12	0.0287	Monacolin L ($C_{19}H_{30}O_4$)
	V13	0.0283	Platensimycin B3 ($C_{23}H_{27}NO_5$)
Dichloromethane and methanol (DCM:M = 9:1)	V14	0.0191	Mollicellin D ($C_{21}H_{21}ClO_6$)
	V15	0.0162	Gancidin W ($C_{11}H_{18}N_2O_2$)
	V16	0.0113	Gymnodimine B ($C_{32}H_{45}NO_5$)
	V17	0.0276	Venturicidin A ($C_{40}H_{66}O_{10}$)
	V18	0.0082	Aspereline F ($C_{46}H_{82}N_{10}O_1$)
	V19	0.0300	Long chained triglyceride fatty acid
	V20	0.0498	Long chained triglyceride fatty acid
	V21	0.0142	Long chained triglyceride fatty acid
	V22	0.0184	Long chained triglyceride fatty acid

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Table 5. Assignment of the ^{1}H and ^{13}C NMR signal of GW at 400 MHz in $CDCl_3$.

Position		Gancidin W. (Helvetica Chimica Acta Vol. 92) (Bin *et al.*, 2009)		SUK10 (Gancidin W)	
		δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	C	-	172.9	-	166.0
2	-	-	-	-	-
3	CH_2	3.54 – 3.51 (*m*)	46.5	3.55 (*m*)	45.8
4	CH_2	1.94 – 1.87 (*m*), 2.06 – 1.99 (*m*)	23.6	1.88 (*m*), 2.02 (*m*)	23.1
5	CH_2	2.35 – 2.31 (*m*), 1.96 – 1.87 (*m*)	29.1	2.33 (*m*), 2.03 (*m*)	29.2
6	H–C	4.15 (br. *s*)	60.3	4.08 (*d*)	59.3
7	C	-	168.9	-	170.0
8	N–H	-	-	6.44 (*s*)	-
9	H–C	4.29 (*t*, *J* = 7.1Hz)	54.7	4.01 (*t*, *J* = 7.1)	53.7
10	CH_2 or H–C	1.99 – 1.94 (*m*), 1.57 – 1.53 (*m*)	39.4	2.03 (*m*), 1.47 (*m*)	39.0
11	H–C, Me or CH_2	2.06 – 2.01 (*m*)	25.7	1.76 (*m*)	25.0
12	Me	1.01 (*d*, *J* = 6.5)	23.4	0.97 (*d*, *J* = 6.5)	23.0
13	Me	0.97 (*d*, *J* = 6.5)	22.2	0.91 (*d*, *J* = 6.5)	22.0

Hz hertz, δ_H coupling constants (J) for H, δ_C coupling constants (J) for C, *s* singlet, *d* doublet, *t* triplet, *m* multiplet, *q* quadruplet, *br* broad.

FIGURE LEGENDS

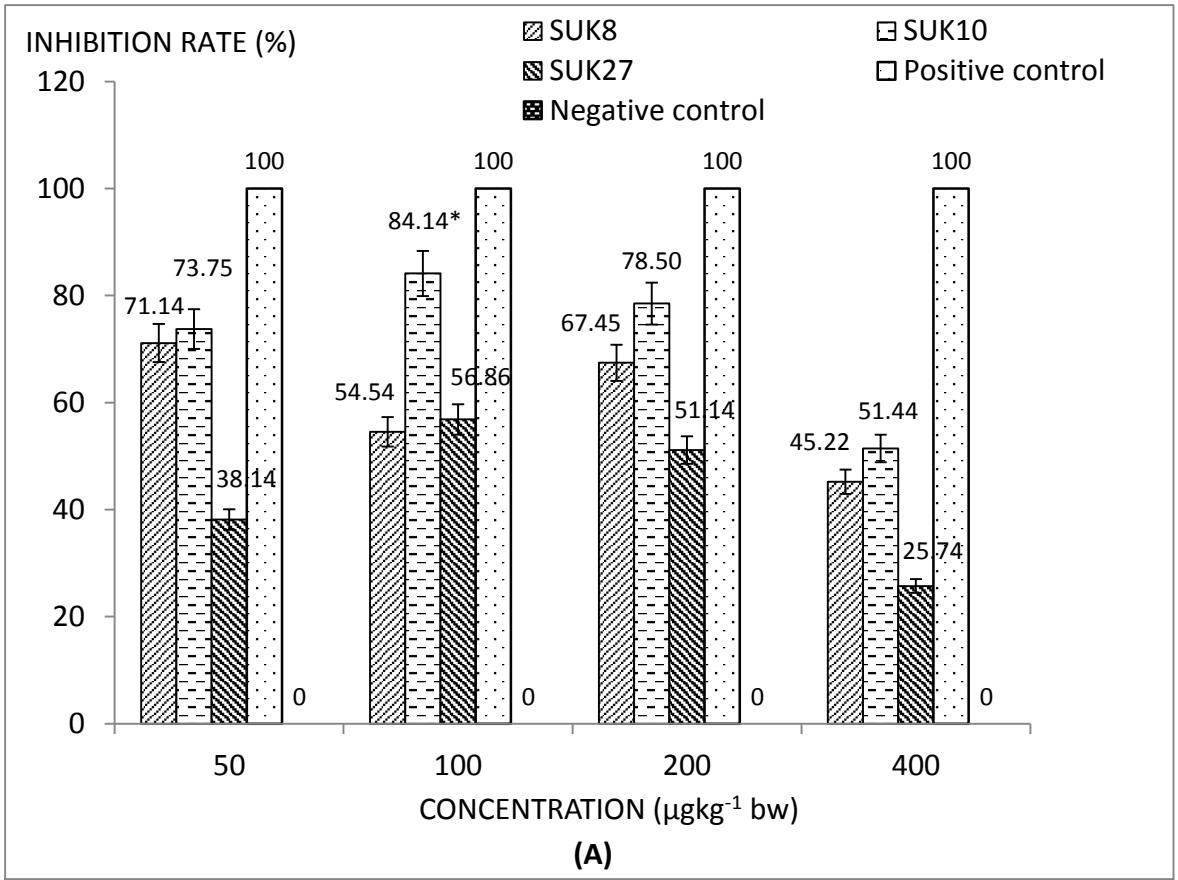
Fig. 1. Inhibition rate (%) on D4 (A) and survival time (day) (B) of mice groups treated with various concentration of ethyl acetate-SUK8, -SUK10 and -SUK27 crude extract, where (*) indicated $p < 0.05$.

Fig. 2. Blood slides taken on D4 post-infection in mice no.1 after four days daily treatment with ethyl acetate-SUK10 crude extract at particular concentration: (A) 5 µg kg^{-1} bw (B) 10 µg kg^{-1} bw (C) 50 µg kg^{-1} bw (D) 100 µg kg^{-1} bw, (E) 200 µg kg^{-1} bw, (F) positive control of 10 mg kg^{-1} bw quinine hydrochloride and (G) negative control of 0.9% normal saline. All four main stages of *P. berghei* lifecycle were successfully captured in the infected-RBC of the mice consisted ring stage (R), trophozoite (T), schizont (S) and gametocyte (G).

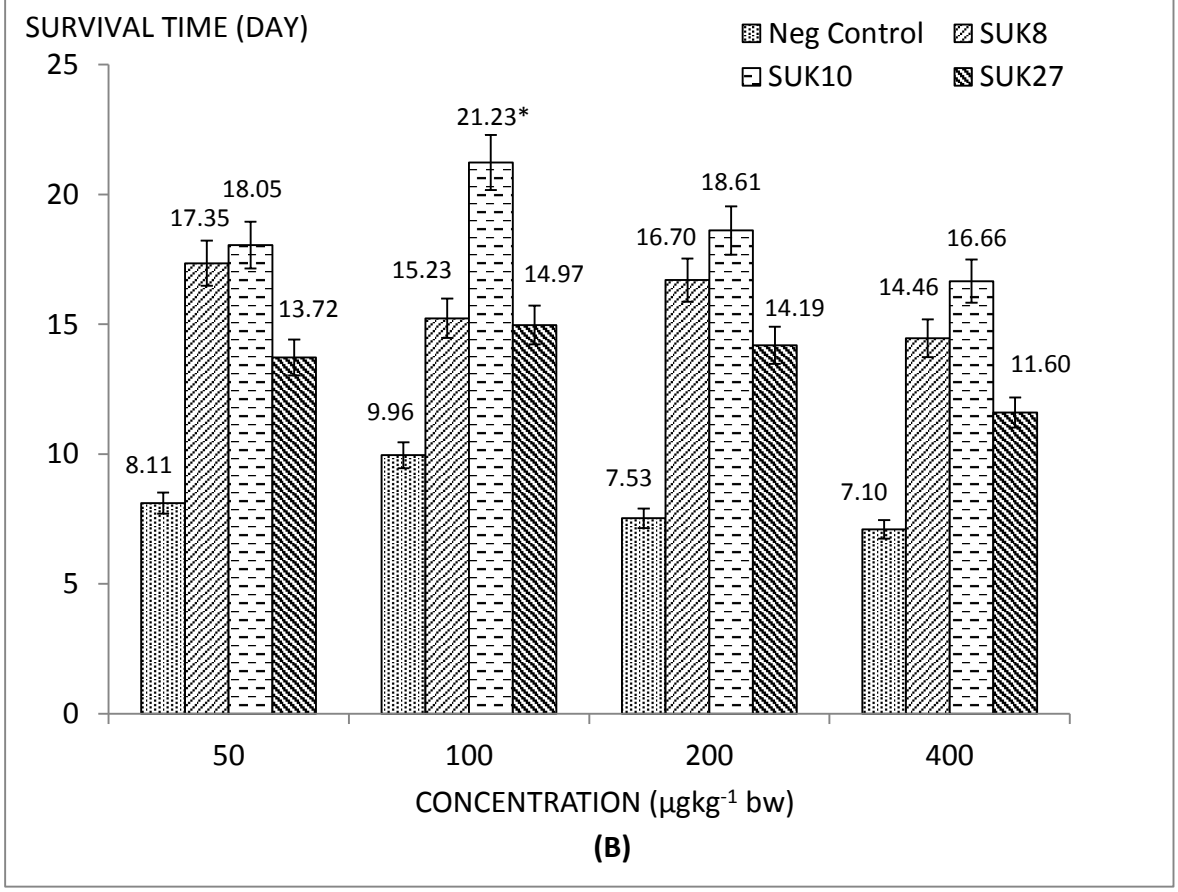
Fig. 3. Inhibition rate (%) on D4 (A) and survival time (day) (B) of mice groups treated with various concentration of ethyl acetate-SUK10, where (*) indicated $p < 0.05$.

Fig. 4. Neighbour joining phylogenetic tree constructed for SUK10 isolate showed that it was felt among the same genus cluster of *Streptomyces* as its genomic sequences is 96 % identical with *Streptomyces hygroscopicus* subsp. *jinggangensis.*

Figure 1


INHIBITION RATE (%)
SUK8
SUK10
SUK27
Positive control
Negative control
120
100
80
60
40
20
0
71.14
73.75
38.14
100
0
54.54
84.14*
56.86
100
0
67.45
78.50
51.14
100
0
45.22
51.44
25.74
100
0
50
100
200
400
CONCENTRATION (µgkg-1 bw)

(A)


SURVIVAL TIME (DAY)
Neg Control
SUK8
SUK10
SUK27
25
20
15
10
5
0
8.11
17.35
18.05
13.72
9.96
15.23
21.23*
14.97
7.53
16.70
18.61
14.19
7.10
14.46
16.66
11.60
50
100
200
400
CONCENTRATION (µgkg-1 bw)

(B)

Figure 2

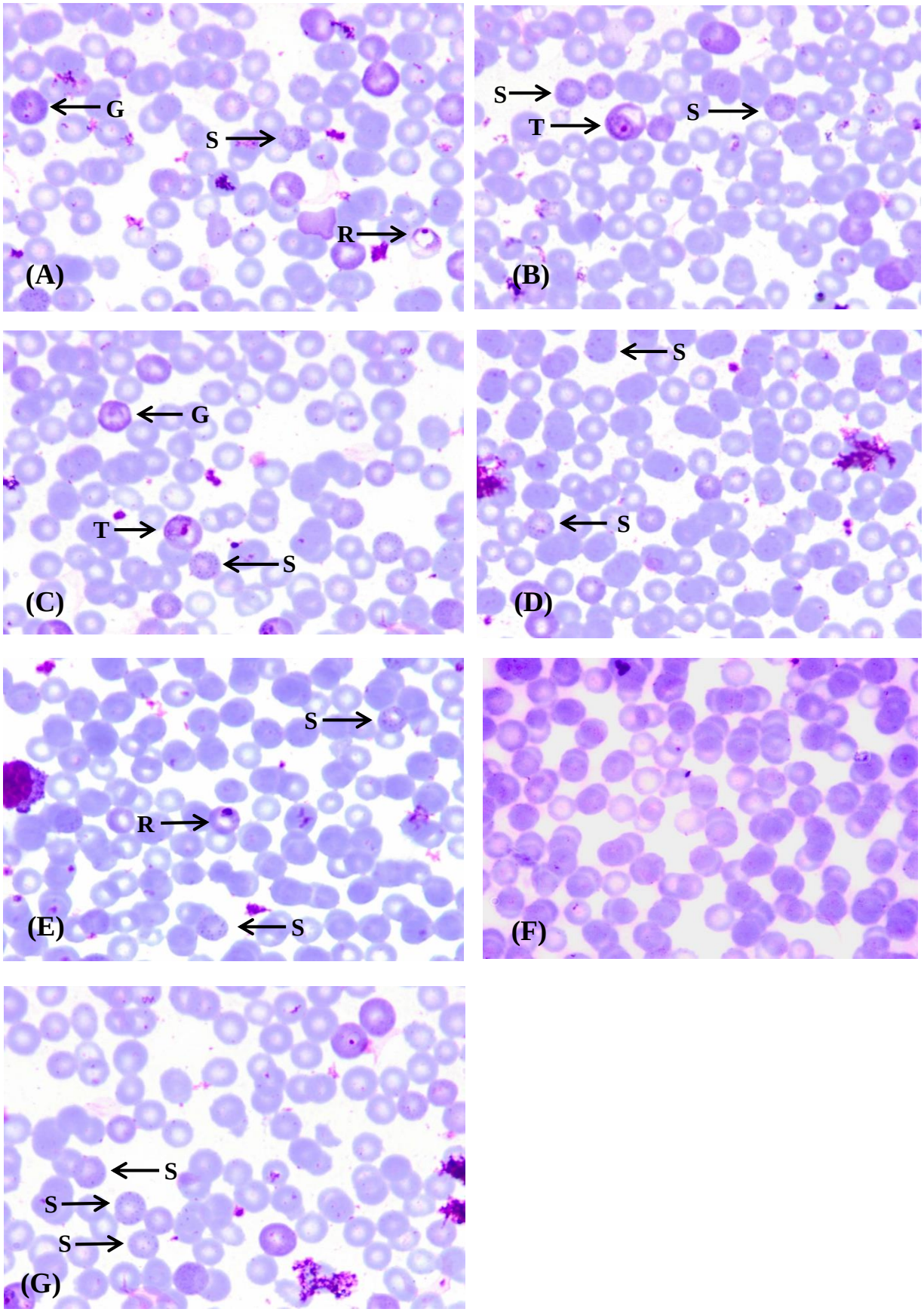
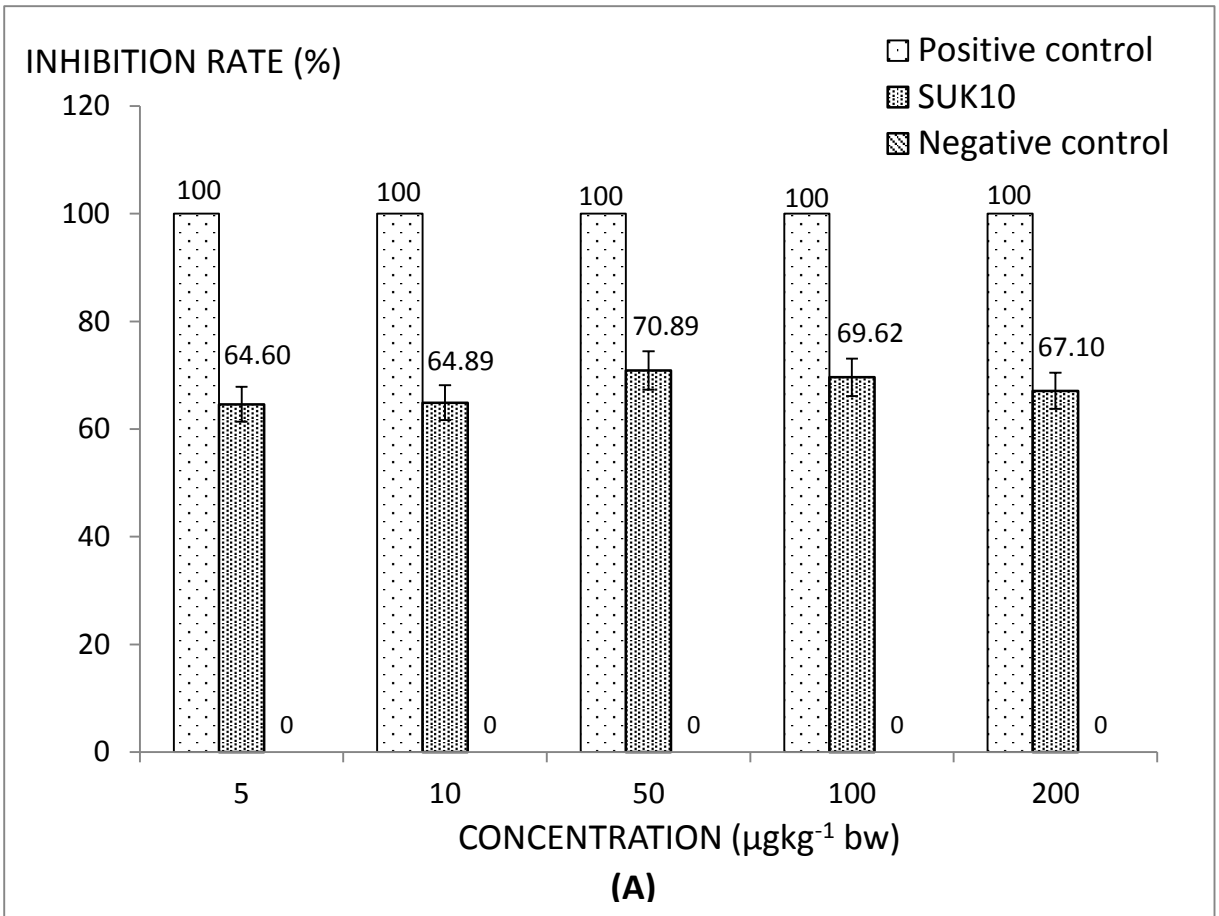
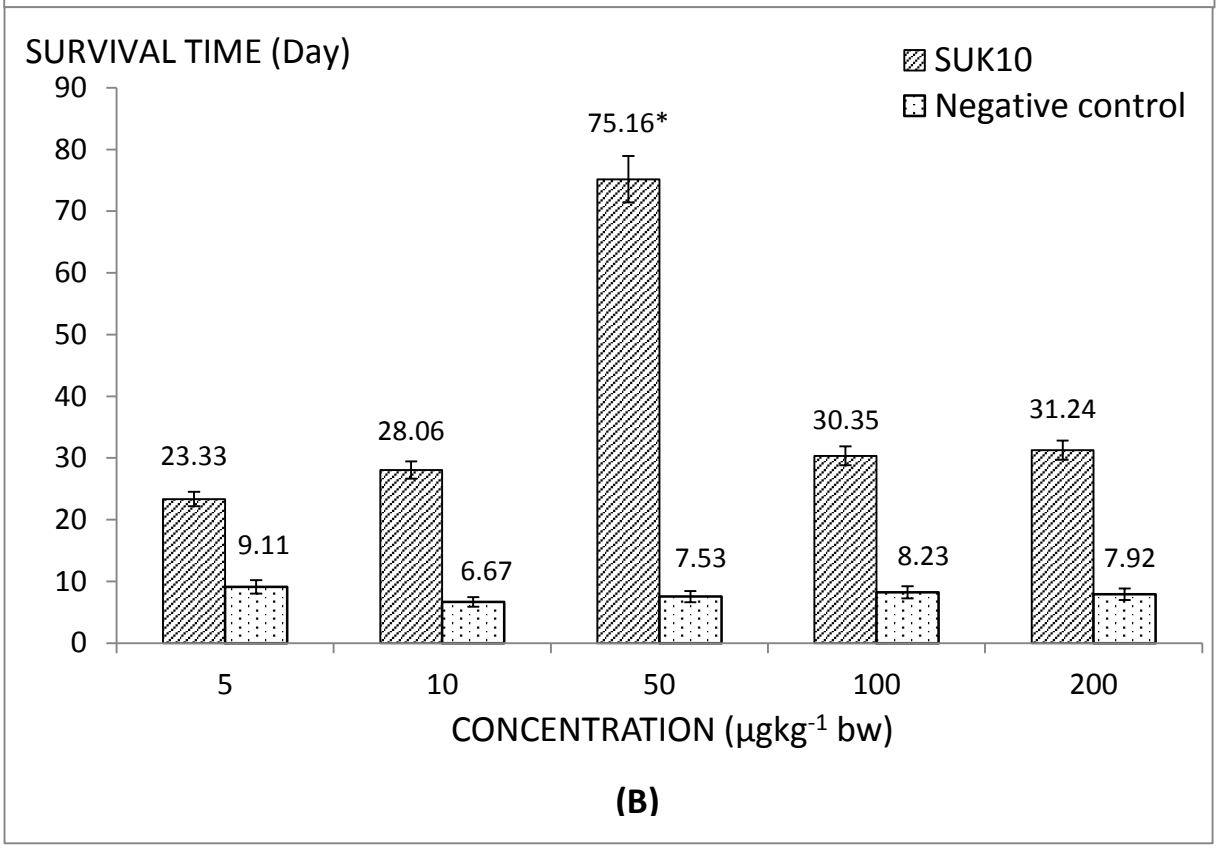


Figure 3

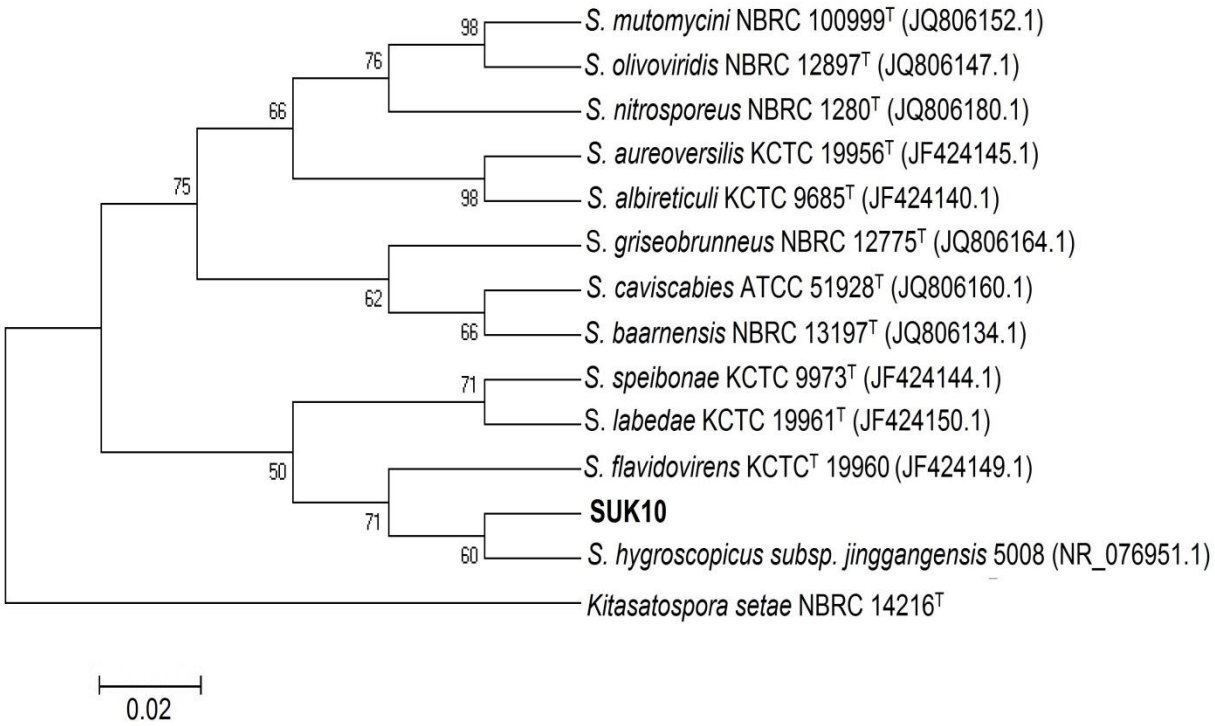

INHIBITION RATE (%)
Positive control
SUK10
Negative control
120
100
80
60
40
20
0
100
64.60
0
100
64.89
0
100
70.89
0
100
69.62
0
100
67.10
0
5
10
50
100
200
CONCENTRATION (µgkg-1 bw)

(A)


SURVIVAL TIME (Day)
SUK10
Negative control
90
80
70
60
50
40
30
20
10
0
23.33
9.11
28.06
6.67
75.16*
7.53
30.35
8.23
31.24
7.92
5
10
50
100
200
CONCENTRATION (µgkg-1 bw)

(B)

Figure 4


98
76
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S. mutomycini NBRC 100999T (JQ806152.1)
S. olivoviridis NBRC 12897T (JQ806147.1)
S. nitrosporeus NBRC 1280T (JQ806180.1)
S. aureoversilis KCTC 19956T (JF424145.1)
S. albireticuli KCTC 9685T (JF424140.1)
S. griseobrunneus NBRC 12775T (JQ806164.1)
S. caviscabies ATCC 51928T (JQ806160.1)
S. baarnensis NBRC 13197T (JQ806134.1)
S. speibonae KCTC 9973T (JF424144.1)
S. labedae KCTC 19961T (JF424150.1)
S. flavidovirens KCTCT 19960 (JF424149.1)
SUK10
S. hygroscopicus subsp. jinggangensis 5008 (NR_076951.1)
Kitasatospora setae NBRC 14216T
0.02

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