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Full Length Research Paper

In vitro evaluation of antitrypanosomal and cytotoxic activities of soil actinobacteria isolated from Malaysian forest

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A total of 83 actinobacteria isolates were successfully revived from cryovials stored in -80°C FRIM actinobacteria culture collection (FACC). Based on macromorphological observation, *Streptomyces*-like group is predominant among all the isolates. The representative actinobacteria isolates were grown in three different fermentation media (M1, M2 and M3) and a total of 249 culture broth extracts obtained were evaluated for *in vitro* antitrypanosomal activity against *Trypanosoma brucei brucei* strain BS221. Five extracts (2.0%) exhibited strong activity (score 3) with an IC_{50} value ≤ 1.56 µg ml^{-1} and 34 extracts (13.7%) exhibited moderate activity (score 2) of 1.56 µg ml^{-1} < IC_{50} ≤ 12.5 µg ml^{-1} . Extracts showing score 3 and score 2 activities were further tested for cytotoxicity. Eight extracts exhibited good selectivity with a SI (Selectivity Index) value ≥ 20. Among the isolates showing good selectivity, isolate FACC-A032 that belonged to the genus *Streptomyces* produced extract with the highest antitrypanosomal activity (IC_{50} = 0.23 µg ml^{-1}) and high selectivity (SI = 76.417). Growth profile study of isolates FACC-A032 in medium M3 exhibited maximum antitrypanosomal activity at day eight of fermentation with IC_{50} = 0.15 µg ml^{-1} and SI = 154.75. This is the first study of *in vitro* antitrypanosomal activity of soil actinobacteria isolated from Malaysian forest.

Key words: Antitrypanosomal, trypanosomiasis, actinobacteria, Alamar blue assay, *Streptomyces.*

INTRODUCTION

African trypanosomiasis causes sleeping sickness in humans and nagana in cattle. This disease is prominent on the World Health Organization (WHO) list of neglected tropical diseases and a major problem to the poorer countries in the world, especially throughout sub-Saharan Africa (WHO, 2010). Chemotherapy, jointly with vector control, remains one of the most important elements in the control of trypanosomatid disease, as there are currently no vaccines to prevent the trypanosome infection. In Malaysia, trypanosomiasis or surra has been reported in institutional farms of cattle (Cheah et al., 1999), rhinocerous centre (Vellayan et al., 2004) and deer breeding centre (Nurulaini et al., 2007; Adrian et al., 2010). The disease was caused by *Trypanosoma evansi* infection. However, there are several cases reported that African trypanosomiasis has appeared in human caused by non-human pathogenic trypanosome species. These species are *T. brucei brucei, Trypanosoma congolense* and *Trypanosoma evansi* (Deborggraeve et al., 2008).

Most pharmaceutical industry has declined their investment in drug development for trypanosomiasis because this disease affects populations who do not represent a profitable market. Thus, only few drugs are currently registered to treat this disease, however, the drugs is limited due to age, toxicity, difficulty to administer, cost and all current treatment suffer from significant drawbacks (Abdel Sattar et al., 2009). Hence,

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new strategies to treat sleeping sickness are urgently required.

Many of our best known and most valuable antibiotics are produced by actinobacteria. It is a prolific producer of structurally diverse bioactive metabolites, and has yielded some of the most important products of the drug industry, including penicillins, aminoglycosides, tetracyclines, cephalosporins and other classess of antibiotics. They have provided over two-thirds of the naturally occurring antibiotics discovered and continue to be a major source of novel and useful compounds (Berdy, 2005). Moreover, Woodruff (1966) proposed that all actinobacteria will produce antibiotics if provided the proper growth conditions.

The actinobacteria are a group of morphologically diverse and Gram-positive bacteria, which comprise a group of branching unicellular microorganisms. Actino-bacteria can be isolated from soil and marine sediments. Soil, in particular is an intensively exploited ecological niche for isolation of actinobacteria that produce many useful biologically active natural products. Among actinobacteria, the genus *Streptomyces* are dominant (Balows et al., 1992). A large number of the commercially and medicinally useful antibiotics have been derived from this genus (Thakur et al., 2007). Several studies reported that actinobacteria have a promising antitrypanosomal activity *in vitro*. (Otoguro et al., 2008; Pimental-Elardo et al., 2010; Zin et al., 2011). Therefore, in the present study 83 isolates of action-bacteria from FACC which was isolated from Malaysian forest soil were studied for potential activity against the trypanosome parasite *T. brucei brucei* strain BS221.

MATERIALS AND METHODS

Soil sample collection, isolation and characterization of actinobacteria

Soil samples were collected from around roots of medicinal and forest plant species at Penang National Park, Malaysia. Soil samples were air-dried at room temperature for 5 to 7 days and were treated using chemical and physical pretreatment methods. Isolation of actinobacteria isolates were done according to the method described by Getha et al. (2004). Actinobacteria colonies were selected from isolates plates and maintained on yeast extract-malt extract (ISP2) agar. Pure cultures were stored as spore suspension in cryovials at -80°C within a FRIM Actinomycetes Culture Collection (FACC). An aliquot of 10 µl culture suspension of 83 actinobacteria isolates from cryovials was transferred to ISP2. Plates were incubated at 28 ± 2°C for seven to ten days until good growth was observed. After the incubation period, all isolates were assigned to the *Streptomyces*-like or non-*Streptomyces* groups based on colony macromorphological characteristics according to methods of Getha et al. (2004)

Fermentation and extract preparation

Three types of production media, M1, M2 and M3 (Getha and Vikineswary, 2002) were prepared and dispensed into many 125-ml Erlenmeyer flasks. Each flask containing 20 ml of medium was plugged with non-absorbent cotton wool and autoclaved at 121°C for 15 min. By using a sterile cork borer, a 5 mm diameter agar-plug was cut aseptically from rich aerial growth of seven to 10-day-old cultures and inoculated into duplicate flasks of each medium. Control flask contained only production medium. The flasks were then incubated at 28 ± 2°C in orbital shaker and shaken at 200 rpm. After seven days of incubation, the whole culture broth was extracted using 1:1 (v/v) butanol (BuOH) according to methods described by Getha et al. (2009). The crude extract was concentrated in a rotary evaporator and about 2 mg of the extracts were dispensed into 96-well microtiter plate and stored at -20°C before use.

Trypanosome parasite and culture medium

T. brucei brucei (*T. b. brucei*) BS221 strain was obtained from the Swiss Tropical Institute, Basel (Jean-Robert et al., 2009). The strain was cultured in minimum essential medium (MEM) with Earle's salts (powder, GIBCO), supplemented with 25 mM HEPES, 1 g L^{-1} additional glucose, 2.2 g l^{-1} $NaHCO_3$, and 10 ml l^{-1} MEM non-essential amino acids (100x). The medium was further supplemented with Balz supplement (Raz et al., 1997), 0.2 mM 2-mercaptoethanol and 15% heat inactivated fetal bovine serum. Fresh supplemented MEM (900 µl) was added into three to four wells of 24-well tissue culture plate and 1:10 dilution was prepared by adding 100 µl of the log phase trypanosome culture to the first well of 24-well plate and was mix well. 100 µl of trypanosome culture from the first well was removed to the second well containing another 900 µl supplemented MEM and the process was repeated to third and fourth well. The log phase of trypanosome culture was visually selected using an inverted microscope. The routine on sub-cultured was done once in a two days and the cultures were incubated at 37°C and 5% CO_2.

In vitro antitrypanosomal assay

The primary, secondary and tertiary *in vitro* antitrypanosomal screening methods were conducted in this study (Hitomi and Kazuhiko, 2005). In the primary assay, two concentrations of actinobacteria extracts (1.56 and 12.5 µg $ml^{-1}l$) were used for the testing. Extracts at each concentration dilution were tested in duplicate plates. Extracts that showed positive for antitrypanosomal activity in both or either one of concentrations well were selected for the secondary and tertiary assays, which consisted of seven final extract concentrations (12.5, 1.56, 0.78, 0.39, 0.19, 0.10 and 0.05 µg ml^{-l}). Extracts were tested in duplicate plates for secondary assay and triplicate plates for tertiary assay. All assays were performed in a flat bottom 96-well microtiter plate. *In vitro* antitrypanosomal activity of test sample was estimated by a dose response curve using Alamar Blue® sensitivity assay according to the method of Raz et al. (1997). Standard trypanocidal drugs, pentamidine isethionate (SIGMA) was dissolved in 5% dimethylsulfoxide (DMSO) and included in the assay as positive control. Negative control-solvent (5% DMSO and 25% ethanol) as well as negative control-blank (Sterile Milli-Q water) were also included in the experiment. Five micro liter of the pre-dilution of the extracts, standard drug and negative control were added to each well of a 96-well microtiter plate. Then, 95 µl of the trypanosomes suspension at a density of 20000 to 25000 trypanosomes ml^{-1} was inoculated to each well except control-blank well. After incubation for 72 h at 37°C under 5% carbon dioxide, 10 µl of fluorescent dye Alamar blue was added to each well and incubation was further continued for 3 to 6 h until colour change is observed. All tests were performed independently two to three times. Plates were analysed

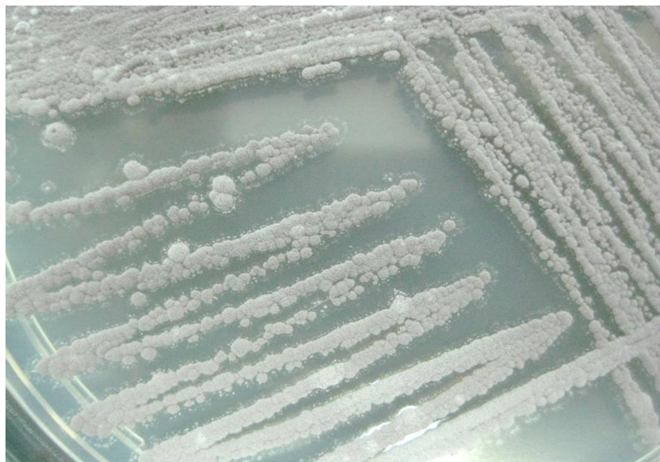

Figure 1a. Culture of *Streptomyces*-like group growing on ISP2 agar.

Figure 1b. Culture of non-*Streptomyces* group growing on ISP2 agar.

using Tecan Infinite M200 plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Absorbance data were transferred into a graphic program (Excel) and were evaluated to determine the IC_{50} values (Hitomi and Kazuhiko, 2005).

Cytotoxicity and selectivity index

In vitro cytotoxicity assay was carried out using normal kidney (Vero) cells according to the procedure described by Kohana and Otoguro (1999) and Malebo et al. (2009). Cells were grown in standard media according to the method reported by Siti Syarifah et al. (2011). Serial dilution of extracts was prepared to produce six final concentrations 0.1, 0.39, 1.56, 6.31, 25.0 and 100 µg ml^{-1} and 10 µl of pre-dilution of extracts, standard drugs, and negative control was added to each well of a 96-well microtiter plate containing of 40000 cells ml^{-1}. Standard drug pentamidine was used as positive control, ethanol and DMSO as negative control-solvent and sterile Milli-Q water as negative control-blank.

The plates were incubated at 37°C under 5% carbon dioxide for 72 h and assayed using the alamar blue assay as described previously with shortened incubation time. After incubation, plates were read using an excitation wavelength of 530 nm and an emission wavelength of 590 nm using the fluorescent plate reader (Tecan Infinite M200) and Magellan software. IC_{50} was defined as the concentration of actinobacteria extract required to reduce a 50% of cell growth compared to control cultures. Based on the cytotoxicity results, calculation of selectivity index (SI) value was performed to select extracts that were very selective to trypanosome parasites and had low toxicity effects on normal cells by using the formula:

$$\text{Selectivity index (SI)} = \frac{IC_{50}\text{ value (cytotoxicity)}}{IC_{50}\text{ value (antitrypanosomal activity)}}$$

Growth profile study of potential actinobacteria isolate

The selected actinobacteria isolate was cultivated in medium M3 for ten days to study the maximum antitrypanosomal activity of active isolate during the fermentation period. Triplicate flasks for each isolate were harvested and analysed each day to observe the culture pH, wet weight of biomass and antitrypanosomal activity. The pH of the fermentation broth was measured with a stainless steel pH meter (IQ 150). Biomass production was expressed as wet weight of cells in 10 ml aliquots of well-mixed whole fermentation broth. The broth was centrifuged at 10,000 rpm for 20 min and the supernatant was discarded with the last drop was blot on a tissue paper and the pellet was then weighed. The remaining 10 ml aliquots of the fermentation broth at each day of fermentation started from day 0 were extracted using 1:1 (v/v) butanol. Crude extract was re-dissolved in ethanol and serially diluted of actinobacteria extracts from day 0 to day 10 of fermentation was then tested for second screening antitrypanosomal activity. Data on pH, wet weighed biomass and antitrypanosomal activity was recorded and plot into growth profile graph.

RESULTS

Macromorphology, fermentation and extraction of actinobacteria isolates

From the total of 83 isolates studied, 45 isolates (54%) belong to the group *Streptomyces*-like and 39 isolates (46%) to the group non-*Streptomyces*. The genus *Streptomyces* is common in actinobacteria population (Masayuki, 2008) and a large number of bioactive compounds are derived from *Streptomyces* species (Berdy, 2005). Isolates falling under *Streptomyces*-like group (Figure 1a) that are fast growers on ISP2 agar were observed after seven days of incubation. Whereas isolates under the non-*Streptomyces*, group (Figure 1b) grew slowly on ISP2 agar and can only be detected after 10 to 14 days incubation. Cultivation of these isolates in different production media was used to optimize expression of bioactive metabolites to produce high-quality extracts (Getha et al., 2009). This is also to increase the chance of isolating the active metabolites

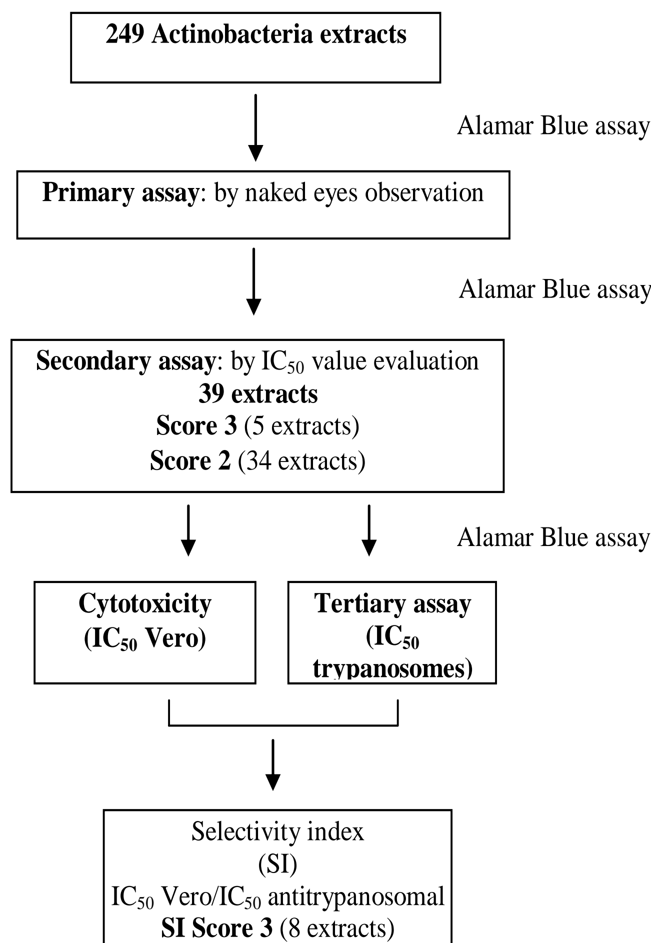

Figure 2. Flow chart showing screening strategy of actinobacteria extracts against trypanosomes and vero to select extracts with potential antitrypanosomal activity.

from actinobacteria. Butanol solvent was used in this study to extract the bioactive compounds from culture broth due to the solvent is most suitable for extraction according to Forar et al. (2007). An easy storage method of large number of extracts in 96-well microtiter plates at -20°C was used in this study before the extracts were tested in bioassay.

Antitrypanosomal activity, cytotoxicity and selectivity index

Antitrypanosomal activity was separated into three categories; IC_{50} > 12.5 µg ml^{-1}: score 1 (low activity); 1.56 µg ml^{-1} < IC_{50} ≤ 12.5 µg ml^{-1}: score 2 (moderate activity) and IC_{50} ≤ 1.56 µg ml^{-1}: score 3 (strong activity). During the primary assay, only two concentrations of extracts were used and the selections of potential extracts were evaluated using naked eye observation of the colour changes in Resazurin. This was a rapid selection method for potential extracts. Rezasurin as a cell proliferation indicator dye (blue colour), will be reduced to a pink fluorescent dye in the medium by cell activity (O'Brien et al., 2000). If there is no cell activity, the blue colour will remain. Therefore when the blue colour appeared in both wells of an extract in the 96-well test plate, this indicates that the extract has a strong positive activity while moderate activity was observed if one of the well shows blue colour. Low activity is observed when both wells show pink colour. The primary and secondary assays showed that 5 extracts (2.0%) demonstrated strong antitrypanosomal activity, 34 extracts (13.7%) exhibited moderate activity and the other extracts showed low or no activity. Extracts that showed strong and moderate activity were selected for the tertiary and cytotoxicity assays to get the selectivity index value (Figure 2).An important criterion in the search of potential compounds against *T. b. brucei* is that they have no or very low toxic effects on mammalian host cell. For this purpose, out of 39 extracts selected after primary and secondary antitrypanosomal assays, the tertiary and cytotoxicity assays showed eight extracts (20.5%) with SI score 3 (SI ≥ 20), seven extracts (17.9%) exhibited SI score 2 (10 < SI < 20) and 24 extracts (61.5%) with SI score 1 (SI ≤ 10). The eight extracts (Table 1) that showed SI ≥ 20 were considered to have good selectivity for the parasite and will be studied for further bioassay guided isolation of the active antitrypanosomal compound/s. Among the eight isolates that produced extracts with good selectivity, one isolate, FACC-A032 exhibited the highest antitrypanosomal activity (IC_{50} = 0.23 µg ml^{-1}) and high selectivity (SI = 76.417) towards the trypanosomae parasite. This isolate was considered for characterization. The morphological, physiological and biochemical characterization indicated that isolate FACC-A032 was assigned to the genus *Streptomyces* (Lili Sahira et al., 2011).

The performance of fermentation media to produced bioactive metabolite in actinobacteria can be strongly influenced by the type of carbon and nitrogen sources (El-Refai et al., 2011). Among the three types of fermentation media used in this study, media M3 that contained the combination of cornstarch and corn steep solids as the carbon and nitrogen sources was found as the most suitable fermentation media for bioactive metabolite production in actinobacteria.

Growth profile of FACC-A032 in medium M3

The growth profile of isolates FACC-A032 in medium M3 is shown in Figure 3. The cell growth reached its maximum (217.59 wet weight/l) on day 3 and the mycelial wet weight of the actinobacteria slightly drop and remain almost constant after four to seven days. As reported by Junker et al. (2009), secondary metabolite fermentation are related with a period of rapid growth and followed by

Table 1. Antitrypanosomal activities of eight actinobacteria extract showing strong activity (Score 3) and moderate activity (Score 2) with high selectivity (SI ≥ 20).

Extract code	IC$_{50}$ value			SI (Vero / BS221)
	Estimated group	Antitrypanosomal (BS221)	Cytotoxicity (Vero)	
A032-M3	STM	0.23 ± 0.014	17.34 ± 0.192	76.417
A026-M3	STM	0.86 ± 0.033	21.02 ± 0.955	24.478
A048-M3	n-STM	1.35 ± 0.046	73.84 ± 0.715	54.616
A049-M3	n-STM	1.40 ± 0.021	> 100	> 71.362
A085-M3	n-STM	4.55 ± 0.072	> 100	> 21.986
A016-M3	STM	3.75 ± 0.022	77.87 ± 0.137	20.7917
A039-M2	n-STM	4.17 ± 0.032	> 100	> 23.965
A052-M3	n-STM	4.69 ± 0.050	> 100	> 21.345
Positive control **(**Pentamidine)		0.00446 ± 0.00006	0.01883 ± 0.00075	4.257

BS221, *Trypanosoma brucei brucei*; Vero, normal kidney cell from African green monkey; STM, *Streptomyces*; n-STM, non-*Streptomyces*; n = 3. All data were recorded as ± standard deviation (SD).

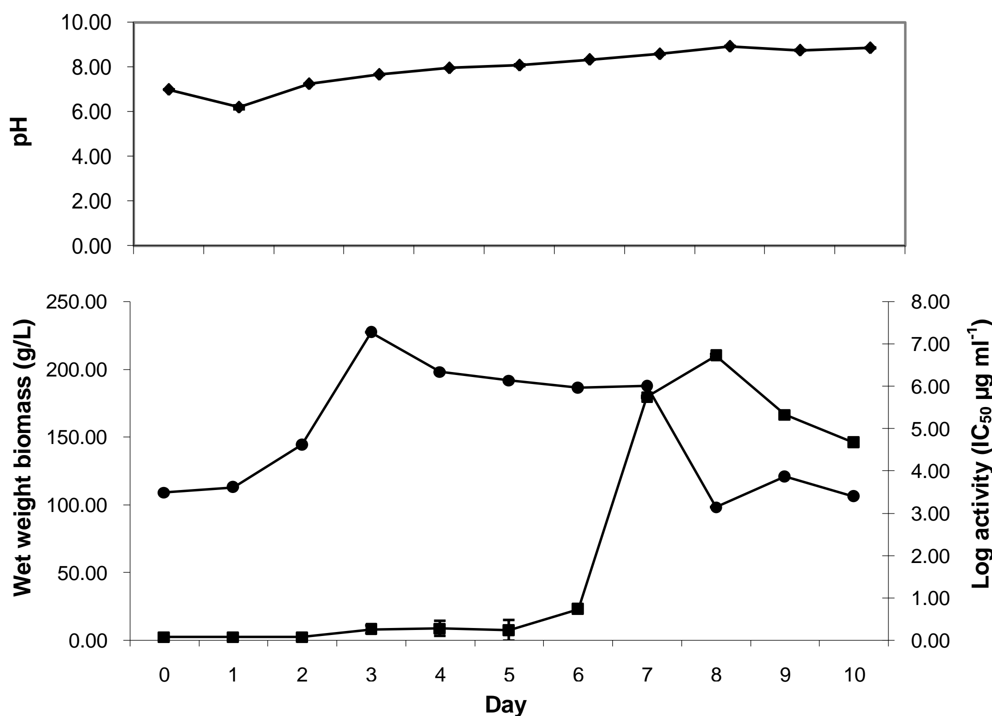

Figure 3. The growth profile of isolates FACC-A032 in medium M3 and their antitrypanosomal activity for ten days. The culture broth was analyzed for the wet weight biomass (•), antitrypanosomal activity (■), and pH (♦). The means ± standard deviations (SD) are display for all data points. Due to low SD, most error bars are not visible.

production phase of secondary metabolite, where production start at the same time with growth rate decline. Based on the previous reported, it was observed that after cell growth decrease on day seven; the antitrypanosomal activity becomes more strongly active. Antitrypanosomal activity was begins on day four of incubation and reach the maximum on day eight. The broth pH profile, which was initially neutral, became alkaline during the growth phase until the end day of fermentation. Results suggest that the maximal antitrypanosomal activity can be found after an optimization of the growth. On the other hand, results from growth profile study will be used as a basis for selection of harvest day (fermentation duration) during large scale

fermentation for bioactive compounds purification.

DISCUSSION

It is known that secondary metabolites produced by actinobacteria possess a wide range of biological activities, and the majority of these compounds are derived from the genus *Streptomyces* (Solanki et al., 2008). Several compounds isolated from actinobacteria have been shown to exhibit antitrypanosomal activity *in vitro*. For example, compounds aureothin, cellocidin, destomycin A, echinomycin, hedamycin, irumamycin, LL-Z 1272β, O-methylnanaomycin A, venturicidin A and virustomycin A were isolated from soil microorganisms and shown to display potent antitrypanosomal activity (Otoguro et al., 2008). Trypanosome parasites GUTat 3.1 (*T.b.brucei*) was used for *in vitro* antitrypanosomal activity. Out of the ten compounds tested, virustomycin A and aureothin showed the highest antitrypanosomal activity, with an IC_{50} value around 0.001 µg ml^{-1}, however aureothin showed the highest SI value > 17,857. Ishiyama et al. (2008) discovered two compounds from soil microorganism KS-505a and alazopeptin, which exhibited antitrypanosomal activity with IC_{50} values of 1.03 and 0.51 µg ml^{-1} respectively, against *T. b. brucei* strain GUTat 3.1. KS-505a show high SI value >27.33 compared to alazopeptin with SI > 9.10. Furthermore, novel antitrypanosomal alkaloid spoxazomicin A-C were isolated from an endophytic actinobacteria, *Streptosporangium oxazolinicum* K07-0460^T as reported by Inahashi et al. (2011) and compounds isolated from *Streptomyces* sp. strains from Mediteranean sponges (valinomycin and staurosporine) show novel antitrypanosomal activity (Pimental-Elardo et al., 2010). Interestingly, isolates FACC-A032 at crude extract level resulted in much lower IC_{50} values of 0.15 µg ml^{-1} after growth profile study was conducted as compared to the KS-505a and alazopeptin. It also showed highest SI with values of 154.75. As reported by Badisa et al. (2009) the higher the SI value, the more selective to trypanosome parasite the extracts are. This paper only discusses antitrypanosomal activity of the crude extracts from actinobacteria, therefore, bioactivity guided isolation can be embarked upon to discover bioactive compound/s from *Streptomyces* sp. FACC-A032 and the other potential isolates from this study. Based on previous literature search, this is the first study to show potential antitrypanosomal activity from a Malaysian soil actinobacteria strain.

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REFERENCES

Abdel Sattar E, Shehab NG, Ichino C, Kiyohara H, Ishiyama A, Otoguro K, Omura S, Yamada H (2009). Antitrypanosomal activity of some pregnane glycosides isolated from *Caralluma* species. Phytomedicine 16:659-664.

Adrian MS, Rehana AS, Latiffah H, Wong MT (2010). Outbreaks of trypanosomiasis and the seroprevalence of *T. evansi* in a deer breeding centre in Perak, Malaysia. Trop. Anim. Health. Prod. 42:145-150

Badisa RB, Darling-Reed, SF, Joseph P, Cooperwood JS, Latinwo LM, Goodman CB (2009). Selective cytotoxic activities of two novel synthetic drugs on human breast carcinoma MCF-7 cells. Anticancer Res. 29:2993-2996.)

Balows A, Trupper HG, Dworkin M, Harder W, Schleifer KH (1992). The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Springer-verlag 1:811-815.

Berdy J (2005). Bioactive microbial metabolites: Review article. J. Antibiot. 58:1-26.

Cheah TS, Sani RA, Chandrawathani P, Sansul B, Dahlan I (1999). Epidemiologi of Trypanosoma evansi infection in crossbred dairy cattle in Malaysia. Trop. Anim. Health. Prod. 31:25-31.

Deborggraeve S, Koffi M, Jamonneau V, Bonsu FA, Queyson, Richard, Simarro PP, Herdewijn P, Buscher P (2008). Molecular analysis of archived blood slides reveals an atypical human *Trypanosoma*. Diagnostic Microbiol. Infect. Dis. 61:428-433.

El-Refai HA, AbdElRahman HY, Abdulla H, Hanna AG, Hashem AH, El-Refai AH, Ahmed EM (2011). Studies on the production of actinomycin by *Nocardioides luteus*, a novel source. Curr. Trends Biotechnol. Pharm. 3:1282-1297.

Forar LR, Ali E, Mahmoud S, Bengra C, Hacene H (2007). Screening, isolation and characterization of a novel antimicrobil producing actinomycete, strain RAF10. Biotechnology 6:489-496.

Getha K, Hatsu M, Wong HJ, Lee SS (2009). Submerge cultivation of basidiomycete fungi associated with root diseases for production of valuable bioactive metabolites. J. Trop. Forest Sci. 21:1-7.

Getha K., Vikineswary S (2002). Antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f.sp. *Cubense* race 4: Indirect evidence for the role of antibiosis in the antagonistic process. J. Ind. Microbiol. Biotechnol. 28:303-310.

Getha K, Vikineswary S, Wong WH, Seki T, Ward A, Goodfellow M (2004). Characterization of selected isolates of indigenous *Streptomyces* species and evaluation of their antifungal activity against selected plant pathogenic fungi. Malaysian J. Sci. 23:37-47.

Hitomi S, Kazuhiko O (2005). *Trypanosoma brucei brucei* In vitro Screening Model. Standard Operating Procedure. p. 11.

Inahashi Y, Iwatsuki M, Ishiyama A, Namatame M, Nishihara-Tsukashima A, Matsumoto A, Hirose T, Sunazuka T, Yamada H, Otoguro K, Takahashi Y, Omura S, Shiomi K (2011). Spoxazomicins A-C, novel antitrypanosomal alkaloids produced by an endophytic actinomycete, Streptosporangium oxazolinicum K07-0460T. J. Antibiot. 64:303-307.

Ishiyama A, Otoguro K, Namatame M, Nishihara A, Furusawa T, Masuma R, Shiomi K, Takahashi Y, Ichimura M, Yamada H, Omura S (2008). *In vitro* and *in vivo* antitrypanosomal activity of two microbial metabolites, KS-505a and Alazopeptin. J. Antibiotics 61:627-632.

Jean-robert I, Reto B, Tanja W, Marcel K, Vanessa Y (2009). Drug screening for kinetoplastids diseases. A training manual for screening in Neglected Diseases. pp. 74.

Junker B, Walker A, Hesse M, Lester M, Christensen J, Connors N (2009). Actinomycetes scale-up for the production of the antibacterial, nocathiacin. Biotechnol. Prog. 25:176-188.

Kohana A, Otoguro K (1999). MRC-5 cells Cytotoxicity evaluation. Standard Operating Procedure. p. 6.

Lili Sahira H, Getha K, Mohd Ilham A, Hema Thopla G, Norhayati I, Muhd Syamil A, Muhd Haffiz J, Siti Syarifah MM, Nur Fairuz MY (2011). Antitrypanosomal activity and characterization of potential actinobacteria isolate. In Raha *et al*. (Eds.) *Leveraging on* MicrobialDiversity for a Sustainable Future Proceedings of International Congress of Malaysian Society for Microbiology, 8-11 December 2011, Penang. pp. 413-416

Malebo HM, Tanja W, Cal M, Swaleh SAM, Omolo MO, Hassanali A, Sequin U, Hamburger M, Brun R, Indiege IO (2009). Antiplasmodial, anti-trypanosomal, anti-leishmanial and cytotoxicity activity of selected Tanzanian medicinal plants. Tanzanian J. Health Res. 11:226-234.

Masayuki H (2008). Studies on the isolation and distribution of rare actinomycetes in soil. Actinomycetologica 22:12-19.

Nurulaini R, Jamnah O, Adnan M, Zaini CM, Khadijah S, Rafiah A, Chandrawathani P (2007). Mortality of domesticated java deer attributed to Surra. Trop. Biomed. 24:67-70.

O'Brien J, Wilson I, Orton T, Pognan F (2000). Investigation of the Alamar Blue (Resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 267:5421-5426.

Otoguro K, Ishiyama A, Namatame M, Nishihara A, Furusawa T, Masuma R, Shiomi K, Takahashi Y, Yamada H, Omura S (2008). Selective and potent *in vitro* antitrypanosomal activities of ten microbial metabolites. J. Antibiotics 61:372-378.

Pimental-elardo SM, Kozytska S, Bugni TS, Ireland CM, Moll H, Hentschel U (2010). Anti-parasitic compounds from *Streptomyces* sp. strains isolated from Mediterranean sponges. Marine drugs 8:373-380.

Raz B, Iten M, Brun YGR (1997). The Alamar Blue® assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. Acta Tropica. 68:139-147.

Siti Syarifah MM, Nurhanan MY, Muhd Haffiz J, Mohd Ilham A, Getha K, Asiah O, Norhayati I, Lili Sahira H, Anee Suryani S (2011).Potential anticancer compound from *Cerbera Odollam*. J. Trop. Forest. Sci. 23:89-96.

Solanki R, Khanna M, Lal R (2008). Bioactive compounds from marine actinomycetes. Indian J. Microbiol. 48:410-431.

Thakur D, Yadav A, Gogoi B, Bora TC (2007). Isolation and scereening of streptomyces in soil of protected forest areas from the states of assam and tripura, India, for antimicrobial metabolites. J. Med. Mycol. 17:242-249.

Vellayan S, Aidi M, Robin WR, Linda JL, Epstein J, Simon AR, Donald EP, Rolfe MR, Terri LR, Thomas JF, Khan M, Vijaya J, Reza S, Abraham M (2004). Trypanosomiasis (Surra) in the captive Sumatran Rhinoceros (*Dicerorhinus sumatrensis sumatrensis*) in Peninsular Malaysia. In Iskandar *et al.* (Eds.) *Animal health: a* breakpoint in economic development? Proceedings of the 16th veterinary Association Malaysia Congress and the 11th International Conference of the Association of Institutions for tropical veterinary medicine, 23-27 August 2004, Selangor. pp. 187-189

WHO (2010). African trypanosomiasis or sleeping sickness. Fact sheet, p. 259.

Woodruff HB (1966). The physiology of antibiotic production: the role of the producing organism. Symp.Soc.Gen.Microbiol. 16:22-46.

Zin NM, Ng KT, Sarmin NM, Getha K, Tan GY (2011). Anti-trypanosomal activity of endophytic Streptomycte. Current. Res. Bacteriol. 4:1-8.