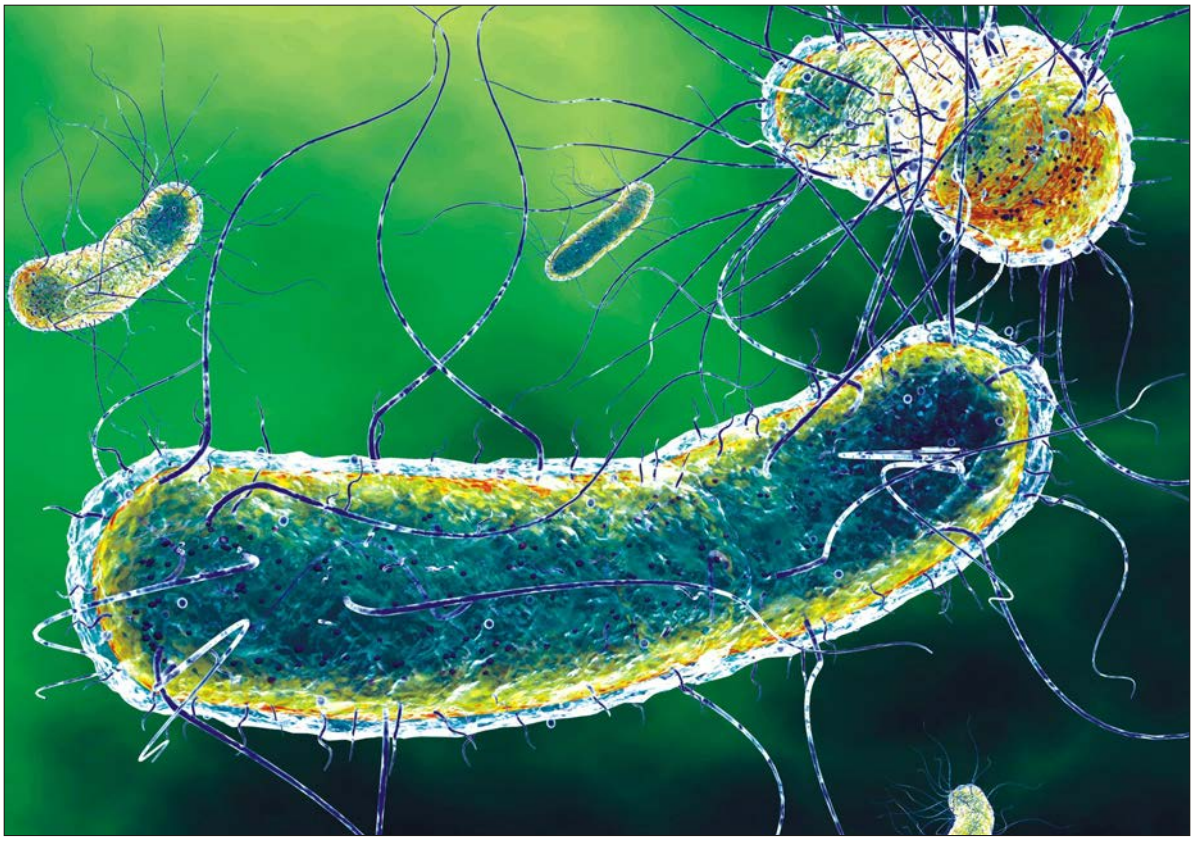

Current Research in Bacteriology

ISSN 1994-5426

science alert

ANSInet
an open access publisher
http://ansinet.com

Current Research in Bacteriology 4 (1): 1-8, 2011
ISSN 1994-5426 / DOI: 10.3923/crb.2011.1.8

Anti-trypanosomal Activity of Endophytic Streptomycete

[1]N.M. Zin, [1]K.T. Ng, [1]N.M. Sarmin, [2]K. Getha and [3]G.Y. Tan
[1]Department of Biomedical Science, Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia
[2]Center of Drug Discovery, Forest Research Institute, 50600, Kuala Lumpur, Malaysia
[3]Institute of Biological Science, University of Malaya, 50603, Kuala Lumpur, Malaysia

Corresponding Author: Noraziah M. Zin, Department of Biomedical Science, Faculty of Allied Health Sciences, Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia

ABSTRACT

This is the first report of anti-trypanosomal activity of endophytic streptomycetes isolated from plants in Malaysia. Eight endophytic streptomycetes were isolated from the stems and roots of *Cinnamomum zeylanicum, Elettariopsis curtisii* and *Zingiber spectabile* which were collected from a Northern Peninsula Malaysia Country Park based on their ethnobotanical properties. The endophytic actinomycetes isolated were subjected to molecular identification whereby DNA was extracted using Sarkosyl, followed by amplification of 16S rRNA using 27f and 765r primer, prior to gene sequencing, BLASTN search and construction of 16S rRNA phylogenetic tree using MEGA4. Simultaneously, secondary metabolites extracted from nutrient broth using ethyl acetate were tested for their anti-trypanosomal activity. The isolates were identified based on their 16S rRNA gene sequences and evaluated for anti-trypanosomal activities. Phylogenetic analyses showed that isolates SUK 17 and SUK 24 were closely related to *Streptomyces niger* strain and the remaining six isolates were closely related to *Streptomyces sampsonii*. Interestingly, although the 16S rRNA gene sequences of both isolates SUK 17 and SUK 24 were 100% identical, only isolate *SUK* 17 which was isolated from *Cinnamomum zeylanicum* has shown positive anti-trypanosomal activity with IC_{50} value of 4.48 μg mL^{-1} for *Trypanosoma brucei brucei* strain BS221 and 60.98 μg mL^{-1} for vero cells with the selectivity index of 13.61. The finding of this research could be a new breakthrough and the discovery of anti-trypanosomal secondary metabolites from isolate SUK 17 may be able contribute to the medical and pharmaceutical field.

Key words: Streptomyces, anti-trypanosomal, rRNA, endophytes, *Trypanosoma brucei*, phylogenetic tree

INTRODUCTION

Human African Trypanosomiasis (HAT), also known as African Sleeping Sickness, is a vector-borne parasitic disease. The parasites are protozoans belonging to the genus *Trypanosoma*, namely *Trypanosoma brucei rhodensiense* which causes Eastern African Trypanosomiasis and *Trypanosoma brucei gambiense* that causes Western African Trypanosomiasis. As a vector, Tsetse fly from the genus *Glossina* transmits the parasites to humans during blood meals (Heelan and Igersoll, 2002; World Health Organization, 2006).

Although the epidemics of trypanosomiasis were more rampant in the past, in the year 2000, World Health Organization (2006) estimated that 60 million Africans were still at risk of

trypanosomiasis. Nevertheless, medical as well as research attention given to this disease remains insufficient. Therefore, WHO categorized HAT as one of the world's most neglected diseases in 2002.

In 2005, due to extensive vector control and strengthened surveillance, there was a substantial reduction in the number of new cases reported per year to 17, 616. However, the current treatment available for late stage trypanosomiasis, namely melarsoprol, remains problematic. Treatment with melarsoprol, an arsenic derivative, has a mortality rate as high as 5% with 19.5% of relapse cases and more than 50% of resistance (Robays *et al*., 2008).

In search for new effective drugs, microbial endophytes have continuously proven to be a promising resource of unique metabolites with great pharmacological potential (Aly *et al*., 2010). Amongst prokaryotes, actinomycetes, notably streptomycetes, remain a rich source of new natural products (Goodfellow and Fiedler, 2010). Endophytic streptomycetes are Gram positive bacteria which for all or part of their life cycle, invade and colonize within the living plant tissues, without causing any apparent adverse effects (Bacon and White, 2000; Wilson, 1995). There are approximately 300, 000 plant species on earth and each plant is host to at least one endophytic microorganism (Strobel and Daisy, 2003). The case that only a few plant species have been thoroughly studied highlights the fact that the opportunity to discover novel and interesting endophytic microorganisms, which may be capable of producing bioactive secondary metabolites, among myriads of plants in a greatly biodiversed ecosystem is astounding (Strobel *et al*., 2005).

Previous research indicated a number of bioactive endophytes were isolated from the actinobacterial phylum, particularly from the genus *Streptomyces* (Coombs and Franco, 2003; Sardi *et al*., 1992; Sessitsch *et al*., 2001). In this study, *Cinnamomum zeylanicum, Elettariopsis curtisii* and *Zingiber spectabile* from Northern Peninsula Malaysia were selected based on their ethnobotanical properties. Attention was given to the filamentous endophytic streptomycetes isolated due to the widely recognized ability of filamentous actinobacterial in producing a spectrum of bioactive molecules. Primarily, endophytic actinomycetes have been isolated and characterized by cultivation-based method followed by molecular analysis. Simultaneously, endophytic actinomycetes were maintained in the laboratory and the secondary metabolites produced by these endophytic actinomycetes were extracted for anti-trypanosomal bioassay.

MATERIALS AND METHODS

Plant selection and location: The plants were selected based on their ethnobotanical properties (Zin *et al*., 2010). Stem and root samples of *Cinnamomum zeylanicum, Elettariopsis curtisii* and *Zingiber spectabile* were collected in 2009 from Bukit Panchor Country Park, Penang Island, Northern Peninsular Malaysia (N 5.14° North latitude and E 100.54°). Specimen voucher numbers were given and the plants samples were submitted to Universiti Kebangsaan Malaysia (UKM) Herbarium collection. The Colonies of endophytic actinomycetes obtained (Zin *et al*., 2010) were subcultured onto International Streptomyces Project 2 (ISP2) agar and incubated at 28°C.

Extraction of endophytic actinomycetes DNA: DNA from the isolates was extracted using a modified Sarkosyl-NaCl protocol (Conn and Franco, 2004). For each isolate, two loopful of mycelium and spores were scrapped from colonies grown on ISP2 medium and suspended in 200 μL of Tris-EDTA (10 mM Tris, 1M EDTA (pH 8.0) by vortexing. To this mixture, 2 μL of RNase (10 mg L^{-1}) was added. The mixture was mixed using vortex and then incubation at 37°C for one hour. Subsequently, 400 μL Solution 1 (1% wt/vol Sarkosyl, 1% wt/vol SDS, 0.5M NaCl) was added with gentle mixing and the mixture incubated at 37°C for 15 min. The mixture was mixed again using a vortex for 5 min and equal volumes of Tris-EDTA-equilibrated phenol chloroform isoamyl

alcohol(25:24:1) was added to it immediately, at room temperature for 30 min with intermittent shaking. The lysates were centrifuged (12,000x g, 10 min) to pellet the cell debris before subsequent extraction with 500 μL chloroform at room temperature for 30 min with intermittent shaking, followed by centrifugation (12,000x g, 15 min). The aqueous layer was precipitated with 3 volumes of cold absolute ethyl alcohol and 0.1 volumes of sodium acetate 3M and was then stored at -20°C overnight. Subsequently, the mixture was centrifuged (12,000x g, 15 min) and the pellet was washed with 70% ethyl alcohol, resuspended in 30 μL sterile distilled water and stored at -20°C. The DNA was semiquantified and visualized using 0.8% agarose gel in half strength of Tris-Borate-EDTA with 2 μL ethidium bromide (0.5 μg mL^{-1}). Simultaneously, the DNA concentration and purity were determined by measuring the intensity of absorbance at wavelengths 260 and 280 nm.

Amplification and sequencing of the 16S rRNA gene: PCR was performed with the universal 16S primer, namely 27f (5'-AGAGTTTGATCMTGGCTCAG -3') and 765r (5'-CTGTTTGCTCCCCACGCTTTC-3') designed to amplify the region between positions 27 and 765 of the 16S rRNA gene in actinobacteria (Conn and Franco, 2004). The 50 μL reactionmixture contained: 5 μM of each primer, dNTPs (10 mM), 1×PCR buffer, 25 mM of $MgCl_2$, 5U *Taq* polymerase and 2 μL template DNA (26-57 ng). The PCR conditions were as follows: initial denaturation at 94°C for 2 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The resultant PCR products were purified by using Wizard PCR Prep DNA purification kit (Promega) before being sent for sequencing Medigene Sdn, Bhd. The automated sequencing temperature cycling profile was identical to the PCR temperature cycling profile. The 16S rRNA sequences obtained were compared to the GenBank databases by using BLASTN.

Phylogenetic analysis: The sequences were aligned using Clustal W packaged in the MEGA 4 (version 4.2) software (Kumar *et al.*, 2001). Phylogenetic analysis was performed with the same software and a Neighbour Joining tree (NJ) was generated with the Kimura two-parameter model (Kumar *et al.*, 2001). Bootstrap analysis was accomplished by using 5,000 replicates.

Fermentation and extraction of secondary metabolites: The secondary metabolites were extracted using a modified protocol (Zin *et al.*, 2007). The isolates were cultured on Bn-2 media, followed by incubation at 28°C for 7 days. A few blocks of Bn-2 media containing streptomycetes were then transferred into 300 mL V-22 broth, followed by incubation at 28°C for 10 days with gentle shaking (140 rpm) using an orbital shaker (Protech, model 720) (Onaka *et al.*, 2005). Subsequently, 30 mL of V-22 broth culture was aseptically transferred into 300 mL of nutrient broth. After 21 days of incubation (Zin *et al.*, 2007), fermentation filtrates were extracted with three half-volumes of ethyl acetate and the crude extracts were separated and dried *in vacuo* at 40°C and 240 mbar.

Anti-trypanosomal assay: Anti-trypanosomal bioassay was carried out using a 96-well microtiter plate protocol with pentamidine as positive drug control and wells without drugs served as negative controls (Getha *et al.*, 2008). Endophytic streptomycete crude extracts at concentrations of 12.5, 1.56, 0.78, 0.39, 0.20 and 0.05 μg mL^{-1} were loaded into the 96-well microtiter plate,. *Trypanosoma brucei brucei* strain BS221 (received from the Swiss Tropical Institute, Basel, Switzerland) at a density of 2.2×10^4 trypanosomes/mL was then inoculated into the 96-well microtiter plate, followed

by 72 h incubation at 37°C in the presence of 5% CO_2. Approximately 10 μL Resazurin 0.0125% was added into each well. This is followed by further incubation for 5 h at 37°C in the presence of 5% CO_2. Background fluorescence and absorbance were determined using fluorescent microplate reader and Magellan V6.4 software at excitation wavelength 528/20 nm and emission wavelength 590/35 with optics positioned on the bottom to determine the IC_{50}. The IC_{50} values were categorized into three scores namely score 1: IC_{50}>12.5 μg mL^{-1}, score 2: 1.56<IC_{50} = 12.5 μg mL^{-1} and score 3: IC_{50} = 1.56 μg mL^{-1}. This bioassay was performed in triplicates.

Cytotoxicity assay: This test was conducted using a 96-well microtitre plate protocol with tamoxifen as the positive drug control and ethanol as negative control (Getha *et al*., 2008). Vero cells at a density of 4.0×10^4 cells mL^{-1} were first inoculated into the 96-well microtitre plate. After 24 h incubation at 37°C in the presence of 5% CO_2, endophytic streptomycetes crude extracts at the concentration of 12.5, 1.56, 0.39, 0.20 and 0.10 μg mL^{-1} were loaded into the 96-well microtitre plate, followed by 72 h incubation. Approximately 10 μL of Resazurin 0.0125% was loaded into the 96-well microtitre plate prior to incubation for 5 h at 37°C. The plates were then analyzed using microplate reader and Magellan v4 software at 492 nm to determine the optical density that indicated the number of living cells. Duplicate plates were used to determine the cytotoxicity of each sample. The Selectivity Index (SI) determined using the following formula, was categorized into 3 scores (Getha *et al*., 2008), namely: score 1: SI = 10 (weak selectivity), score 2: 10 < SI < 20 (moderate selectivity) and score 3: SI = 20 (strong selectivity):

$$\frac{IC_{50}\text{ value (Vero cells)}}{IC_{50}\text{ value (Trypanosoma brucei brucei BS221)}}$$

RESULTS

Partial 16S rRNA sequencing was initially used to identify the actinobacterial endophytes isolated to the genus level and to determine their clusters of similar microorganisms. Comparison with GenBank databases (Table 1) showed all eight actinobacterial isolates were *Streptomyces* spp.

Although the 16S rRNA gene sequences were only about 700 base-pair long, the NJ phylogenetic tree has showed that both SUK 17 and SUK 24 were closely related to *Streptomyces niger* strain AS4.1244,); Isolates 18, 19, 20 and SUK 23 were closely related to *Streptomyces sampsonii* and isolates 21 and SUK 22 were closely related to *Streptomyces sampsonii* strain 173409 (Fig. 1) When the crude extracts of endophytic steptomycetes were tested for anti-trypanosomal activity, only crude extracts from SUK 17 showed positive anti-trypanosomal activity towards *Trypanosoma brucei brucei* strain BS221 with the IC_{50} value of 4.48 μg mL^{-1} (Table 2) and IC_{50} value of 60.98 μg mL^{-1} in cytotoxicity test (Table 3) with Selectivity Index (SI) 13.61.

Table 1: Identification of endophytic actinomycetes isolates based on partial sequencing of the 738-bp region of the 16S rRNA gene

Identification*	% similarity	No. of isolates
S. cheonanensis (AY822606)	100	SUK 17, 24
S. carpaticus (DQ442494)	100	SUK 17, 24
S. niger (FJ406109)	100	SUK 17, 24
S. albidoflavus (FJ591130)	100	SUK 18,19,20, 23
S. sampsonii (AB362247)	100	SUK 18,19,20, 23
S. sampsonii (EU570682)	100	SUK 21, 22

*Nearest match (es)

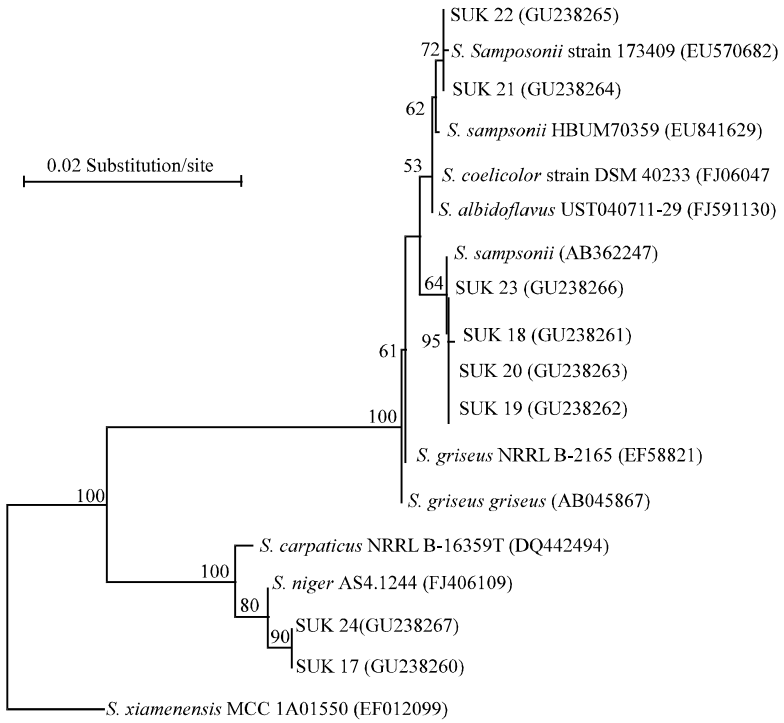

Fig. 1: NJ phylogenetic tree of the partial 16S rRNA sequences from endophytic steptomycetes isolated

Table 2: Determination of anti-trypanosomal activity and IC_{50} of SUK 17 crude extract

Crude extract of SUK 17 (GU238260)		Pentamidine	
Conc. (µg mL^{-1})	Average absorbance	Conc. (ng mL^{-1})	Average absorbance
12.5	6519.67	6.25	0.97790
1.56	41351.33	3.125	0.34165
0.78	43610.33	1.56	0.44688
0.39	40023.00	0.78	0.46301
0.20	40761.67	0.39	0.46392
0.10	42070.33	0.20	0.46527
0.05	41035.67		
IC_{50} (µg mL^{-1})	4.48	IC_{50} (ng mL^{-1})	4.14

Table 3: Determination of cytotoxicity and IC_{50} of SUK 17 crude extract.

Conc.(µg mL^{-1})	Average absorbance
12.5	667.50
1.56	3195.50
0.78	3210.00
0.39	3096.00
0.20	2992.00
0.10	3012.50
IC_{50} (µg mL^{-1})	60.98

DISCUSSION

In present laboratory, endophytic steptomycetes were primarily isolated and characterized by cultivation-based method (Zin *et al.*, 2010) followed by DNA extraction and subsequent molecular analysis (Ghadin *et al.*, 2008). Three antagonistic actinomycetes were successfully identified based on morphological, physiological and colour formation on different agar by Muiru *et al.* (2008). This approach was selected due to the fact that identification of non-culturable actinobacterial endophytes did not give apparent contribution to our research.

The Neighbour Joining phylogenetic tree showed that both isolates 17 and SUK 24 were closely related and the alignment of 16S rRNA gene sequences of both isolates showed 100% similarity to *Streptomyces niger* strain AS4.1244. However, this finding remains inconclusive. In order to increase its reliability and validity, the more than 1,400 base-pair of the 16S rRNA gene sequence should be analyzed along with the phenotypic features (Shirling and Gottlieb, 1966) as well as biochemical data (Cross and Goodfellow, 1973) such as cell wall composition. Nevertheless, interestingly, only isolate SUK 17 isolated from *Cinnamomum zeylanicum* showed positive anti-trypanosomal activity and SUK 24 isolated from *Elettariopsis curtisii* did not show similar activity. This finding suggested that the production of anti-trypanosomal secondary metabolites could be strain-specific. The concept that novel species may contain unique compound as the evolution of secondary metabolites may act as a driver for bacterial speciation (Czaran *et al.*, 2002; Jensen, 2010). The antitrypanosomal assays showed that there was difference between the IC_{50} value of SUK 17 crude extract (4.48 μg mL^{-1}) and pentamidine (4.14 ng mL^{-1}).

The extract showed a positive antitrypanosomal activity of score 2 ($1.56 < IC_{50} = 12.5$ μg mL^{-1}). The calculation of Selectivity Index (SI) was performed to select extracts that are very selective to trypanosome parasites but show low toxicity against normal cells. The *SUK* 17 crude extract showed moderate selectivity of score 2 (SI = 13.61) to the trypanosome parasites. Aly *et al.* (2011) have reported of using metabolites from endophytic fungus *Penicillium* sp., as antitrypanosomal,

In most cases, secondary metabolites are produced extracellularly. However, there are possibilities whereby the active compounds are produced intracellularly such as those produced within mycelia. In order to assess such compounds, mycelia should be subjected to chemical extraction. However, it was observed in this study that formation of mycelia in nutrient broth was not significant. Therefore, the culture filtrate of fermentation broth has been used and subjected to the extraction procedure (Zin *et al.*, 2007).

Apart from *SUK* 24, other *SUK* isolates did not show positive anti-trypanosomal activity. The reasons may be multifactorial. Each of these secondary metabolite classes has their own characteristics such as molecule structures, active groups and polarity indexes. In this study, ethyl acetate, with polarity index 4.4, was used as solvent to extract the secondary metabolites from the fermentation filtrate. Under such extraction condition, only secondary metabolites which are soluble in ethyl acetate will be extracted and other compounds will not be extracted efficiently even though the compounds may have certain levels of anti-trypanosomal activity.

Apart from factors mentioned above, other factors like prolonged absence of endophytes in natural hosts as well as minimum competition may be contributors to negative result. Generally, those ecosystems having the greatest biodiversity seem to be the ones also having the most biodiversed endophytes (Strobel and Daisy, 2003). This is because in such an ecosystem, competition is great, resources are limited and selection pressure is at its peak. Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists in

ecosystems where the evolutionary race to survive is the most active. In the laboratory, on the other hand, the competition from other microorganisms is insignificant. Abundant resources with minimum selection pressure and naturally, the genes responsible for production of the secondary metabolites to sustain their survival will be suppressed partly, if not entirely.

This preliminary study is still at its early stages of discovery and development of potential lead compounds from microbial sources in the search of treatment for Human African Trypanosomiasis. Although further studies are required, the discovery of secondary metabolites from endophytic streptomycetes that show positive antitrypanosomal activity in this study, undeniably, remains an interesting finding which ultimately may contribute to mankind.

ACKNOWLEDGMENTS

This study was supported by the Research University Grant of Universiti Kebangsaan Malaysia (UKM-GUP-TKP-08-22-074). The authors would like to thank Kitasato Institute, Japan for their valuable advice and Ms Lili Sahira from Forest Research Institute Malaysia for her technical assistance in the antitrypanosomal studies.

REFERENCES

Aly, A.H., A. Debbab, J. Kjer and P. Proksch, 2010. Fungal endophytes from higher plants: A prolific source of phytochemicals and other bioactive natural products. Fungal Diversity, 41: 1-16.

Aly, A.H., A. Debbab, C. Clements, R. Edrada-Ebel and B. Orlikova *et al*., 2011. NF ê B inhibitors and antitrypanosomal metabolites from endophytic fungus *Penicillium* sp. Isolated from *Limonium tubilorum*. Bioorg. Med. Chem., 19: 414-421.

Bacon, C.W. and J.F. White, 2000. Microbial Endophytes. Marcel Dekker, New York.

Conn, V.M. and C.M.M. Franco, 2004. Analysis of the endophytic actinobacterial population in roots of wheat (*Triticum aestivum* L.) by terminal restriction fragments length polymorphism and sequencing of 16S rRNA Clones. Applied Environ. Microbiol., 70: 1787-1794.

Coombs, J.T. and C.M.M. Franco, 2003. Isolation and identification of actinobacterial isolated from surface-sterilized wheat roots. Applied Environ.. Microbiol., 69: 5303-5308.

Cross, T. and M. Goodfellow, 1973. Taxonomy and classification of actinomycetes. Soc. Applied Bacteriol. Symp. Ser., 2: 11-112.

Czaran T.L., R.E. Hoekstra and L. Page, 2002. Chemical warfare between microbes promotes biodiversity. Proc. Natl. Acad. Sci. USA., 99: 786-790.

Getha, K., L.S. Husin, M.I. Adenan, O. Kazuhiko, M. Namatame, A. Nishihara and T. Furisawa, 2008. Diversity of actinomycetes from penang national park and their antitrypanosomal activity. Proceedings of the 30th Symposium Malaysian Society for Microbiology, Aug. 16-19, Hyatt Regency Resort Kuantan, Pahang, Malaysia, pp: 522-526.

Ghadin, N., N.M. Zin, V. Sabaratnam, N. Badya, D.F. Basri, H.H. Lian and N.M. Sidik, 2008. Isolation and characterization of a novel endophytic streptomyces SUK 06 with antimicrobial activity from malaysian plant. Asian J. Plant Sci., 7: 189-194.

Goodfellow, M. and H.P. Fiedler, 2010. A guide to successful bioprospecting: Informed by actinobacterial systematic. Antonie van Leeuwenhoek, 98: 119-142.

Heelan, J.S. and F.W. Ingersoll, 2002. Essentials of Human Parasitology. Delmar Thomson Learning, USA., pp: 97.

Jensen, P.R., 2010. Linking species concepts to natural product discovery in the post-genomic era. J. Ind. Microbiol. Biotechnol., 37: 219-224.

Kumar, S., K. Tamura, I.B. Jakobsen and N. Masatoshi, 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics, 17: 1244-1245.

Muiru, W.M., E.W. Mutitu and D.M. Mukunya, 2008. Identification of selected actinomycete isolates and characterization of their antibiotic metabolites. J. Boil. Sci., 8: 1021-1026.

Onaka, H., M. Nakaho, K. Hayashi, Y. Igarashi and T. Furumai, 2005. Cloning and characterization of goadsporin biosynthetic gene cluster from *Streptomyces* sp. TP-A0584. J. Microbiol., 151: 3923-3933.

Robays, J., G. Nyamowala, C. Sese and V.B.K.M. Kande, P. Lutumba, W. van der Veken and M. Boelaert, 2008. High failure rates of Melarsoprol for sleeping sickness, Democratic Republic of Congo. Emerg Infect. Dis., 14: 966-967.

Sardi, P., M. Saracchi, B. Quaroni, G.E. Borgonovi and S. Merli, 1992. Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. Applied Environ. Microbiol., 58: 2691-2693.

Sessitsch, A., B. Reiter, U. Pferfer and E. Wilhelm, 2001. Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and actinomycetes-specific PCR of 16S rRNA genes. FEMS Microbiol. Ecol., 1305: 1-10.

Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol., 16: 313-340.

Strobel, G. and B. Daisy, 2003. Bioprospecting for microbial endophytes and their natural products microbiol. Microbiol. Mol. Biol. Rev., 67: 491-502.

Strobel, G., B. Daisy and U. Castillo, 2005. Novel Natural Products From Rainforest Endophytes. In: Natural Products Drug Discovery and Therapeutic Medicine, Zhang, L. and A.L. Demain (Eds.). Humana Press, New Jersey, pp: 329-351.

Wilson, D., 1995. Endophyte-the evolution of a term and clarification of its use and definition. Oikos, 73: 274-276.

World Health Organization, 2006. Weekly Epidemiological Record. World Health Organization, Rome, Italy, pp: 469-480.

Zin, N.M., M.N.I. Sarmin, N. Ghadin, D.F. Basri, N.M. Sidik, W.M. Hess and G.A. Strobel, 2007. Bioactive endophytic *Streptomycetes* from Malay Peninsula. FEMS. Microbiol. Lett., 274: 83-88.

Zin, N.M., C.S. Loi, N.M. Sarmin and A.N. Rosli, 2010. Cultivation-dependent characterization of endophytic actinomycetes. Res. J. Microbiol., 5: 717-724.