Endophytic fungi from Maliau Basin, Sabah: Identification of strains and antimicrobial compounds

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ABSTRACT

Aims: Endophytic fungi are microorganisms that live asymptomatically within plant tissues, producing a wide range of metabolites, including compounds potentially useful for drug development. We investigated endophytic fungi from Maliau Basin, Sabah to identify strains producing bioactive compounds, notably with antimicrobial activity.

Methodology and results: A total of 23 plants were sampled yielding 345 endophytic fungal isolates. Of these, 44 isolates were screened for antimicrobial activity against nine species of bacteria and fungi, revealing 14 endophytes producing bioactive metabolites. Crude fungal extracts were obtained from broth cultures of endophytic isolates with promising activity while the fungal strains were identified using molecular methods. The crude extract of endophyte MB4 WA10, isolated from *Callophyllum* sp. (bintangor) showed IC_{50} of 2.6 mg/mL against *S. aureus* and 0.6 mg/mL against *B. subtilis* while the extract of MB22 WA16, an isolate identified as *Valsaceae* sp., was also active against *S. aureus* with an IC_{50} of 1.37 mg/mL. Another isolate, namely MB5 L4 (WA), was identified as a *Phomopsis* sp. and its extract was the most active against *S. aureus* with an IC_{50} of 1 mg/mL. The HPLC fraction of this fungal extract with the highest inhibition (92.37%) of *S. aureus* was purified for compound isolation and identification. A polyketide compound, 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one ($C_9H_{12}O_3$), with molecular weight of 168.192 was identified based on mass spectral and NMR data analysis. This previously identified compound is known to have other antimicrobial properties.

Conclusion, significance and impact of study: Rainforests in Malaysia, especially Maliau Basin, harbour many species of fungal endophytes, producing useful bioactive compounds that may be explored for further potential uses, including antimicrobial activity.

Keywords: Phomopsis, *Valsaceae*, antimicrobial, endophytic fungi, Maliau Basin

INTRODUCTION

Microorganisms are well-known for producing many biologically active chemicals. These bioactive compounds have contributed vastly to drug development research. For instance, they are often used as lead structures for drug discovery. Endophytic fungi, which asymptomatically inhabit plants, produce a wide range of bioactive compounds, such as terpenoids, xanthones, phenols, benzopyranones, cytochalasins, enniatins and many others (Schulz *et al.*, 2002). These endophytes are, therefore, potentially new sources for future pharmaceutical development, as well as industrial production. Although endophytic fungi are found in many plant hosts, their diversity and biological activities vary among plant species. Owing to the various benefits of endophytic fungi especially to human health (Zhao *et al.*, 2011), it is therefore worth investigating inhibitory

activities of endophytic fungal metabolites against health-threatening microorganisms.

Microbial infections and antimicrobial resistance are currently recognised as major problems for human health and animal farming. There is thus a pressing need for discovering new sources of antimicrobial lead compounds. Endophytic fungi possess prominent antibacterial, antifungal and herbicidal activities (Schulz *et al.*, 2002). For instance, Phongpaichit *et al.* (2006) reported that fungal endophytes isolated from the plant *Garcinia* sp. showed promising antimicrobial effects against bacteria, including *Staphylococcus aureus* and fungi such as *Candida albicans*, *Cryptococcus neoformans* and *Microsporum gypseum*. Interestingly, cytosporone U extracted from an endophytic *Phomopsis* sp. exhibited potent inhibitory effect against tobacco mosaic virus (Tan *et al.*, 2017). A literature review by Deshmukh *et al.* (2015) listed 265 antibacterial

compounds that were isolated from endophytic fungi since the year 2000. Endophytic fungi are, therefore, undoubtedly important sources for the discovery of many potent antimicrobial molecules.

The Malaysian rainforests house a tremendous variety of plants and the Maliau Basin Conservation Area in Sabah is a pristine, highly biodiverse forested area with some 1806 species of higher plants recorded (Hazebroek *et al.*, 2004). Such a wide variety of plants in an undisturbed ecosystem may provide some interesting endophytes. In this study, we aimed to identify endophytic fungal strains isolated from plants in the Maliau Basin, evaluate their overall antimicrobial activity and identify the antimicrobial compounds of interest isolated from the fungal strains.

MATERIALS AND METHODS

Plant sampling

A total of 23 plant samples were collected from the Maliau Basin Lowland Mixed Dipterocarps Forest. Plant parts including the leaves, stems and roots were observed for endophytic fungi.

Isolation of endophytic fungi

Plant samples were cleaned with running water and surface sterilised by dipping into a series of bleach solution, ethanol and sterile distilled water (Bills, 1996). The samples were cut into small parts (0.5-1 cm^2) and plated onto potato dextrose agar and water agar (Merck). Emerging fungal isolates were transferred to potato dextrose agar to obtain a pure culture.

Screening for antimicrobial activity

Fungal isolates were screened against a panel of target microorganisms, which included the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and the fungi *Candida albicans*, *Aspergillus fumigatus*, *A. niger*, *Fusarium solani and Trichoderma viridae* obtained from the culture collection at Novel Antibiotics Laboratory, Biomedical Science Programme, Faculty of Health Sciences, Universiti Kebangsaan Malaysia. For initial screening, the endophytes were cultured on potato dextrose agar plates for 1-2 weeks at room temperature (27 °C) and the pathogens were streaked around the endophyte. Any growth inhibition of the target pathogens was observed for up to 3 days. Alternatively, endophytes were cultured in 100 mL potato dextrose broth, for 2 weeks and 50 µL of broth was evaluated for antimicrobial metabolites in an agar well diffusion assay. The broth was directly placed in the well made on nutrient agar plates streaked with the target microorganism and any inhibition zone around the well was observed for up to 3 days.

Fungal extract preparation

Endophytic fungi (isolates MB4 WA10, MB5 L4 (WA), MB22 WA16) were cultured onto potato dextrose agar for 1 week. Two agar plugs (0.5 cm × 0.5 cm) were cut, transferred to 300 mL potato dextrose broth in a 1-L flask and incubated for 2 weeks at room temperature (approx. 27 °C), with shaking. Broth cultures were filtered to remove the mycelia. The remaining broth was extracted using ethyl acetate (Merck) at a 1:1 (v/v) broth to solvent ratio. The ethyl acetate partition was dried by rotary evaporation to produce a crude extract.

Determination of IC$_{50}$ of crude extracts

Crude extracts at concentrations of up to 5 mg/mL were dissolved in 5% (v/v) methanol and filter-sterilised. The extracts were used in agar well diffusion assay to determine the presence of antimicrobial activity against microorganisms that were inhibited in the screening assay. Following this the IC$_{50}$ of crude extracts was determined using a microtitre plate assay. Each well of a microtitre plate was added with 5 µL extract (serially diluted), 40 µL test microorganism (5 × 10^5 CFU/mL for bacteria) and 50 µL medium (Mueller Hinton broth for bacteria). After 24 h incubation at 27 °C, 20 µL MTT solution (5 mg/mL) was added to all wells and incubated for 4 h following which the formazan product was solubilised in 100 µL dimethyl sulfoxide. The viability of bacterial cells was calculated by measuring the absorbance of the test well at 540 nm and subtracting the absorbance of cell free control (solvent solution + medium). This gave the cell viability (%) in each well treated with an analyte. The absorbance of cell free control was considered as 0% growth reference and the analyte free control (solvent solution + test organisms in medium) was considered as 100% growth reference. The percentage inhibition of cell growth in each well was calculated as 100% - cell viability %. The IC$_{50}$ of the extract was determined at 50% cell growth inhibition.

HPLC profiling and fractionation

Dried extracts were dissolved in HPLC grade methanol at 10 mg/mL concentration and analysed by HPLC (Waters Alliance e2695 Separation Module, USA equipped with Waters 2998 Photodiode Array Detector and Waters Symmetry C18 column (250 mm × 10 mm i.d., 5 µm). Detection was done at 254 nm wavelength. The mobile phase consisted of aqueous solution of (A) water with 0.1% (v/v) formic acid and (B) 100% (v/v) methanol. The gradient system was used, starting with 10% to 90% B in 20 min and reversed from 90% to 10% in 10 min. The flow rate of the mobile phase was set at 1 mL/min and the sample injection volume was 100 µL. Ten mg of crude extract was separated into 80 semi-purified fractions via HPLC and dried using the Minivac centrifugal evaporator. Fractions were tested against the target microorganisms for antimicrobial activity.

Table 1: Antimicrobial activity of endophytic fungal isolates, crude extracts and HPLC fractions.

Plant source	Endophytic fungus (Isolate code)	Microorganisms inhibited	IC$_{50}$ of crude extracts	Inhibitory activity of HPLC fractions
Bintangor (*Callophyllum* sp.)	MB4 WA10	*S. aureus* *B. subtilis*	IC$_{50}$ = 2.6 mg/mL IC$_{50}$ = 0.62 mg/mL	No active fraction No active fraction
Monsit (*Zizyphus angustifolios*)	MB5 L4 (WA)	*S. aureus* *B. subtilis*	IC$_{50}$ = 0.99 mg/mL IC$_{50}$ = 0.5 mg/mL	2 active fractions No active fraction
Cengal (*Neobalanocarpus heimii*)	MB22 WA16	*S. aureus*	IC$_{50}$ = 1.37 mg/mL	No active fraction

Identification of bioactive compound

Following determination of antimicrobial activity of HPLC fractions, the fraction showing the highest bioactivity was selected for compound isolation. The fraction was collected using the same HPLC method that was used for crude extract fractionation. The bioactive fraction was manually collected referring to its retention time and UV profile. The HPLC process and fraction collection was performed several times so that the quantity of compound obtained was sufficient for structure elucidation. The collected fraction was concentrated by application of nitrogen gas while the sample was on a heating block (30 °C).

To identify the compound in the bioactive fraction, the purified fraction was solubilised in deuterated chloroform (CDCl$_3$) and analysed by nuclear magnetic resonance (NMR) spectroscopy using Bruker Avance III Spectrometer equipped with cryoprobe at the frequency of 600 MHz and 150 MHz for one-dimensional (1D) ^{1}H-NMR and ^{13}C-NMR and 2D-NMR analyses such as correlation spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC) and heteronuclear multiple-bond coherence (HMBC). In addition to the NMR studies, liquid chromatography/mass spectrometry (LC/ESI-MS) analysis was performed using Bruker MicroToF-Q, USA in a positive-ion mode. The spectral data obtained were compared with those included in the AntiBase database. All data were consistent with those previously published (Figure 1; Osterhage, 2001).

NMR and MS data

NMR and MS data for the identified compound is as follows:
^{1}H NMR (CDCl$_3$, 600 MHz) δ 6.87 (1H, dq, *J*= 15.6, 7.2 Hz), 6.37 (1H, dq, *J*= 15.6, 1.8 Hz), 2.98 (3H, d, *J*= 1.8 Hz), 1.73 (3H, s), 1.55 (3H, s); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 202.6, 177.0, 139.8, 119.2, 107.2, 102.1, 22.5, 19.3, 5.7; HRESIMS *m/z* 169.0870 [M+H]$^+$ (calcd for C$_9$H$_{13}$O$_3$ 169.0864).

Molecular identification of fungal isolates

To identify the bioactive endophytic fungal strains, fungal DNA was extracted from Whatman FTA card as previously described (Hanif *et al.*, 2018). Amplification of the ribosomal RNA gene internal transcribed spacer (ITS) sequence by PCR using the primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS2 (5' GCT GCG TTC TTC ATC GAT GC 3') was followed by sequencing of amplified products by 1st Base (Seri Kembangan, Malaysia). The DNA sequences were analysed using BLAST (Basic Local Alignment Search Tool) on the NCBI website (https://blast.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

From 23 plant species that were sampled in Maliau Basin, that included climbers (*Piper* spp.), trees (*Callophyllum* sp.) and hardwood trees (cengal), a total of 345 endophytic fungal isolates were obtained and categorised into 142 morphotypes based on features such as texture, pigmentation and growth rate of cultures on potato dextrose agar. The initial screening of selected endophytes from slow-growing morphotype groups (44 isolates) revealed antibacterial activity for 11.3% of isolates tested (5 isolates), antifungal activity for 11.3% of isolates (5 isolates), while 9% (4 isolates) were active
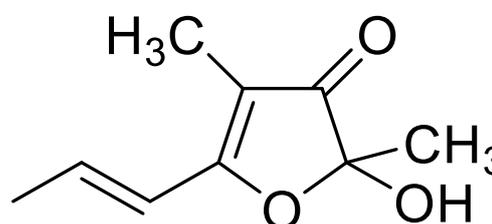

Figure 1: The structure of 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-*trans*-propenylfuran-3-one.
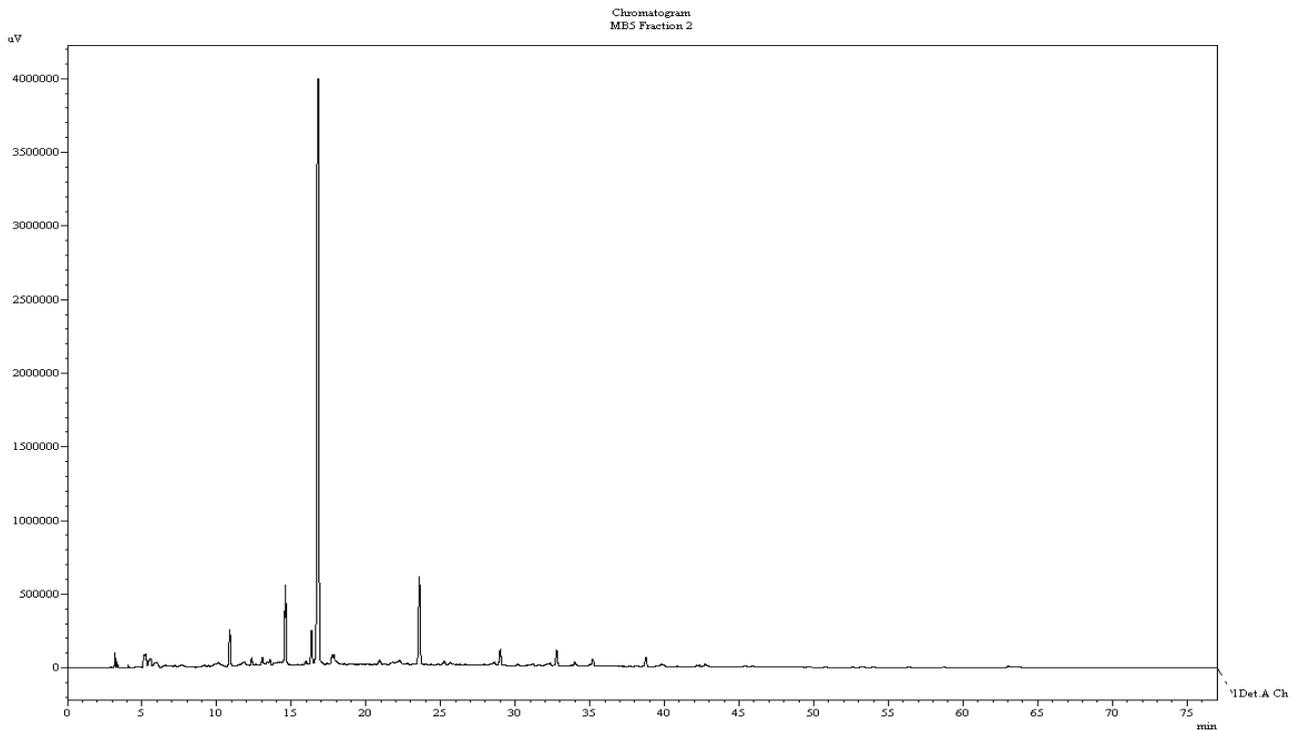
Figure 2: Chromatogram of MB5 L4 (WA) fungal extract showing a major peak at the 17th min.
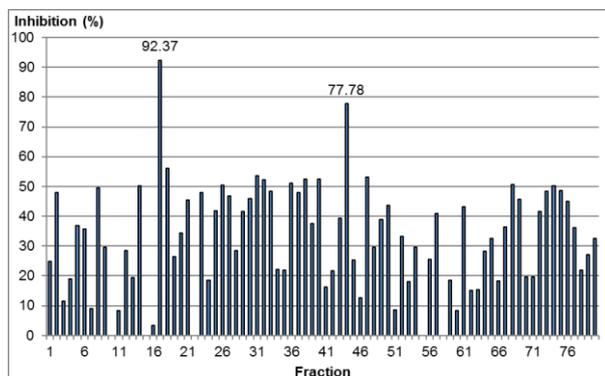

Figure 3: Inhibitory activity of MB5 L4 (WA) semi pure fractions against *S. aureus* with the highest inhibition shown by the 17th and 44th fractions.

against both bacteria and fungi (Supplementary Data). Of these 14 active isolates, two endophytes (MB5 L4 (WA) and MB22 WA16) that showed a broad range of antimicrobial activity against bacteria and fungi and one (MB4 WA10) which showed activity against *S. aureus* and *B. subtilis* were selected for identification of fungal strains, extraction and identification of fungal metabolites and analysis of antimicrobial activity of the extracted bioactive compounds.

The crude ethyl acetate extract of MB5 L4 (WA) isolated from the leaf of a creeper plant, commonly known as monsit (*Zizyphus angustifolios*) only inhibited the growth of *S. aureus* and *B. subtilis* while the extract of

MB22 WA16 isolated from cengal (*Neobalanocarpus heimii*) leaf was active against only *S. aureus* (Table 1). The endophyte MB4 WA10, isolated from *Callophyllum* sp. (bintangor) leaf, showed consistent antibacterial activity against *S. aureus* and *B. subtilis.* The IC_{50} of the crude extracts ranged from 0.5 mg/mL to 2.6 mg/mL. In the search for potential antimicrobial compounds, crude extracts with MIC < 200 µg/mL are sought (Hormazabal and Piontelli, 2009). Therefore, the endophyte extracts appeared to have weak antibacterial activity.

Due to greater antibacterial activity against *S. aureus,* the dried crude extract of MB5 L4 (WA) was separated into 80 semi pure fractions from 10 mg crude extract injected to the HPLC system. All fractions were tested against *S. aureus* to determine the bioactive fraction(s) in view of identifying the bioactive compound(s).

From the UV profile of MB5 L4 (WA) extract at 254 nm, a major peak was observed at the 17th min, which corresponds to the 17th fraction collected (Figure 2). All the 80 fractions collected were evaluated for bioactivity against *S. aureus* and displayed varying levels of inhibition (Figure 3). The fractions collected at the 17th min and 44th min showed the highest activity with 92.37% and 77.78% inhibition of *S. aureus*, respectively. However, the fraction collected at the 44th min did not show any peak under 254 nm UV detection. Therefore, only the 17th fraction was processed for compound identification in this fraction. The compound from MB5 L4 (WA) fraction at the 17th min (17th fraction) was

Table 2: Molecular identity of endophytic fungal isolates based on internal transcribed spacer (ITS) sequence.

Endophytic fungus (Isolate code)	Isolate identity	GenBank Accession number	Closest match in Genbank (Accession no.)	Nucleotide homology	Query coverage
MB5 L4 (WA)	*Phomopsis* sp.	MH204107	*Phomopsis bougainvilleicola* (JX847139)	91%	100%
MB22 WA16	*Valsaceae* sp.	MH204108	*Valsaceae* sp. (AB334109)	91%	100%

identified as 2,3-dihydro-2-hydroxy-2,4-dimethyl-5-trans-propenylfuran-3-one based on analysis of mass and NMR data (Figure 1). This polyketide compound has the molecular formula of $C_9H_{12}O_3$, with molecular weight of 168.192 and accurate mass of 168.078645. It was previously determined to exhibit minor antifungal, antiplasmodial effect and inhibition of tyrosine kinase suggesting anti-tumour effect (Osterhage *et al.*, 2000). This compound was previously isolated from other fungal species, namely the phytopathogenic fungus *Stemphylium radicinum* (Grove, 1971), the algal endophyte *Ascochyta saliconiae* (Osterhage *et al.*, 2000), endophyte *Mollisia nigrescens* from blueberry (Ibrahim *et al.*, 2017) and the soil-inhabiting fungus *Virgaria boninensis* (Ishii *et al.*, 2015). As this compound exhibits mild and non-specific activity in a range of assays, it is therefore of limited therapeutic potential.

In the initial antimicrobial screening of the endophytic isolates, a wider spectrum of antimicrobial activity was observed compared to the activity of the crude ethyl acetate extracts obtained from fungal broth cultures. The variation is mainly caused by the extraction process that is limited by the solubility of the bioactive compounds in the solvent used. Ethyl acetate is suitable for extraction of mid-polar compounds like polyketides, peptides and other amino acid derivatives (Vanmiddlesworth and Cannell, 1998; Hamann, 2006). Ethyl acetate is not miscible with aqueous solutions, therefore it can be used to extract secreted compounds in fungal broth culture without freeze drying the broth. In order to extract the maximum number of metabolites from fungal culture, various solvents may be used, including methanol which is a polar solvent that dissolves a wide range of compounds. In addition, endophytic fungi often lose the ability to produce bioactive metabolites when repeatedly sub-cultured in axenic culture which may be due to a lack of host-specific stimuli (Deepika *et al.*, 2016). To stimulate the production of secondary metabolites, various methods have been employed, including co-culture with other microorganisms such as *B. subtilis* (Ola *et al.*, 2013) culture on natural medium (Ola *et al.*, 2013; Kamauchi *et al.*, 2016) and genetic modulations (Deepika *et al.*, 2016).

Molecular identification of MB5 L4 (WA) isolate based on the fungal ITS region showed closest identity (91%) with *Phomopsis bougainvilleicola* (JX847139, Cariello *et al.*, 2013), therefore this isolate is most likely a *Phomopsis* sp. For isolate MB22 WA16, it showed 91% identity with *Valsaceae* sp. (AB334109, Osono *et al.*, 2008). The query coverage for both isolates was 100%

with the respective matched subjects (Table 2). Both *Phomopsis* spp. and *Valsaceae* spp. have been often isolated as endophytic fungi from various locations and plant sources worldwide, but most isolates were not identified to the species level. An identity of >97% and a query coverage >90% has been deemed significant for phylogenetic determination of fungal species (Cariello *et al.*, 2013); therefore, our isolates may represent novel species. Furthermore, both *Phomopsis* (Tan *et al.*, 2017) and *Valsaceae* (Higginbotham *et al.*, 2014) were reported to possess antiviral and antiparasitic functions, respectively. In this light, this study provides early evidence on the potential of endophytic fungi isolated from Maliau Basin as a source of antimicrobial compounds.

CONCLUSION

We conclude that the rainforest in Malaysia harbours many useful endophytes, awaiting discovery. Antimicrobial compounds are one example of pharmacologically useful products of endophytic fungi. A greater understanding of secondary metabolite gene expression and regulation in fungi will enable enhanced production of bioactive compounds in these genetically diverse microorganisms.

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SUPPLEMENTARY DATA

Antimicrobial activity of endophytic fungal isolates detected during initial screening. https://drive.google.com/file/d/1dqfOo9vZ-IvaNQFSSw-bQ8nGUyobR93l/view?usp=sharing

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