Research article

Cytotoxic and antibacterial activities of endophytic fungi isolated from plants at the National Park, Pahang, Malaysia

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Abstract

Background: Endophytes, microorganisms which reside in plant tissues, have potential in producing novel metabolites for exploitation in medicine. Cytotoxic and antibacterial activities of a total of 300 endophytic fungi were investigated.

Methods: Endophytic fungi were isolated from various parts of 43 plants from the National Park Pahang, Malaysia. Extracts from solid state culture were tested for cytotoxicity against a number of cancer cell lines using the MTT assay. Antibacterial activity was determined using the disc diffusion method.

Results: A total of 300 endophytes were isolated from various parts of plants from the National Park, Pahang. 3.3% of extracts showed potent (IC₅₀ < 0.01 μg/ml) cytotoxic activity against the murine leukemic P388 cell line and 1.7% against a human chronic myeloid leukemic cell line K562. Sporothrix sp. (KK29FL1) isolated from Costus speciosus showed strong cytotoxicity against colorectal carcinoma (HCT116) and human breast adenocarcinoma (MCF7) cell lines with IC₅₀ values of 0.05 μg/ml and 0.02 μg/ml, respectively. Antibacterial activity was demonstrated for 8% of the extracts.

Conclusion: Results indicate the potential for production of bioactive agents from endophytes of the tropical rainforest flora.

Background

Endophytes are microbial entities that live within living tissues of plants without apparently any deleterious consequences [1]. Their biological diversity, especially in temperate and tropical rainforests, is large. Each plant species may be host to a number of endophytes [2]. Since the discovery of the world's first billion-dollar anticancer compound - paclitaxel (Taxol) - could be biosyn-
thesized by Pestalotiopsis microspora, a fungus that colonizes the Himalayan yew tree, interest in studying such endophytes for their medicinal potential has grown tremendously [3]. To date, endophytes have been most extensively studied for their ability to produce antibacterial, antiviral, anticancer, antioxidants, antidiabetic and immunosuppressive compounds [1]. Their study is expected to become an important component in the production of new natural bioactive products.

Only a few studies on endophytic fungi from Malaysian plant species have been conducted so far. The current study was undertaken to investigate this biodiversity and to isolate and screen endophytic fungi with cytotoxic and antibacterial activities from medicinal plants collected from two locations in the National Park, Pahang, Malaysia.

**Methods**

**Source of endophytic fungi**

Plant materials were obtained from the National Park, Pahang, Malaysia in June, 2007. Two different locations, Kuala Keniam (KK) and Kuala Trenggan (KT), where medicinal plants could be found in abundance were selected for sampling. Chosen parts from individual plants were collected and stored at 4°C until used. All plant samples were identified by Kamaruddin Saleh of the Forest Research Institute of Malaysia (FRIM) and were deposited in the herbarium at the Faculty of Pharmacy, Universiti Teknologi MARA, Shah Alam, Malaysia.

**Isolation of endophytic fungi**

Isolation of endophytes from the 43 plant samples was carried out as described by Strobel et al., [4] but with minor modifications. Plant samples, which included leaves, stems, roots, rhizomes, flowers, fruits and bark, were washed under running tap water for 10 min followed by immersion in 70% EtOH for 1 min and in NaOCl (2.5% - 5.25%) for 3 min, drained and immersed in 70% EtOH again for 30 sec. Finally, the samples were rinsed with sterile dH2O. Each plant sample was cut aseptically into 1 cm long segments. The cut surfaces of the segments were placed on petri dishes containing potato dextrose agar (PDA) (Oxoid) supplemented with chlorotetracycline HCl (50 μg/ml, Sigma) and streptomycin sulphate (250 μg/ml, Sigma) at 28°C. Pure cultures were then transferred to PDA plates free of antibiotics and maintained in the culture collection of the Collaborative Drug Discovery Research (CDDR) Group, UiTM, Malaysia. For investigations of biological activity, the endophytes were cultivated for 14 days on PDA plates at 28°C.

**Semipolar extraction of fungal cultures**

Crude endophytic extracts were prepared as described by Lang et al., [5] but with slight modifications. Endophytic cultures (five plates per fungus) were homogenized and transferred to a 500 ml conical flask filled with 250 ml EtOAc (Merck) and left to stir overnight at room temperature. The mixture was filtered through Whatman No.1 filter paper, after which Na2SO4 (40 μg/ml, Merck) was added to further remove the aqueous layer within the mixture. The mixture was then transferred to a round bottom flask and dried using a rotary evaporator. The resultant extract was dissolved in 1 ml of dimethyl-sulfoxide (DMSO) (Sigma) and kept at 4°C as stock solution.

**Cytotoxic activity**

Human chronic myeloid leukemic, K562 (ATCC CCL - 243), murine leukemic, P388 (ATCC TIB 63), human colorectal carcinoma, HCT116 (ATCC CCL - 247) and human breast adenocarcinoma, MCF7 (ATCC HTB - 22) cell lines were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. All cell lines were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories) and 1% penicillin/streptomycin (PAA Laboratories). Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂.

Cytotoxicity of extracts at various concentrations (0.01 - 100 μg/ml) was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma) assay, as described by Mosmann, 1983 [6] but with minor modification, following 72 h of incubation. Assay plates were read using a spectrophotometer at 520 nm. Data generated were used to plot a dose-response curve of which the concentration of extract required to kill 50% of cell population (IC₅₀) was determined. Cisplatin (Mayne Pharma) and tamoxifen (Dynapharm), which are both established chemotherapeutics, were used for comparison. Cytotoxic activity was expressed as the mean IC₅₀ (± standard deviation) of three independent experiments.

**Antibacterial activity**

The crude extracts of the 300 endophytic fungi were tested against Bacillus subtilis (ATCC 6633), Micrococcus luteus (ATCC 10240), Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853). Antibacterial activity was determined using the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCCLS) [7]. Pre-warmed Mueller-Hinton agar (MHA) (Oxoid) plates were seeded with 10⁷ - 10⁸ cfu suspension of test bacteria. Endophytic extracts (10 μl) dissolved in DMSO (1 mg/ml) were pipetted (10 μl) onto sterile paper discs (6 mm diameter, Oxoid) and placed onto the surface of inoculated agar plates. Gentamicin sulphate (10 μg, Oxoid) was used as the positive control. Plates were incubated at 37°C for 48 h. Antibacterial activity was expressed as the
diameter of the inhibition zone (mm) produced by the extracts.

Results and discussion
A total of 300 endophytes were isolated from 43 plants found at two different locations (Kuala Keniam and Kuala Trenggan) within the National Park, Pahang, Malaysia (Table 1). Of the total endophytes obtained, 70.0% were isolated from plants at Kuala Keniam, and the remaining from Kuala Trenggan. Relatively greater distribution of endophytes was found within leaf (48.7%), stem (25.7%) and root (16.3%) samples compared to other segments (9.3%, including flower, fruit, rhizome and bark) of the plants. *Ardisia colorata* (laloh, local name) was found to

| Plant code* | Plant species                      | No. of isolates obtained from: | Total |
|-------------|-----------------------------------|--------------------------------|-------|
| KK1         | Donax grandis Ridl.               | 2 2 1 5                        |       |
| KK2         | Angiopteris evecta (G. Forst.) Hoffm. | 2 2 1 5                      |       |
| KK3         | Clidemia hirta (L.) D. Don        | 5 3 3 11                       |       |
| KK4         | Palmae sp.                        | 3 2                            |       |
| KK5         | Anisochotolype mollisima Hassk.    | 4 2 1 2                       | 9     |
| KK6         | Cnestis palola (Lour.) Merr.       | 3 4 1                          | 8     |
| KK7         | Sindora coriacea (Baker) Prain.   | 5                              | 2 7   |
| KK8         | Antias toxicaria (Pers.) Lesch     | 6                              | 6     |
| KK9         | Phylogenath rotundifolios (Jack) Blume | 3 6 3 12                   |       |
| KK10        | Catunaregam spinosa (Thunb.) Tirveng | 3 2 2 7                     |       |
| KK11        | Unidentified                      | 3                              | 3     |
| KK12        | Tacca integrifolios Ker Gawl.     | 4 2 3                          | 9     |
| KK13        | Ixora grandiflora Zoll. & Mor.    | 2                              | 1 3   |
| KK14        | Ampelocissus cinnamomea Planch.   | 3 5 1                          | 9     |
| KK15        | Unidentified                      | 3                              | 1     |
| KK16        | Tetracera indica Merr.            | 3 2                            | 5     |
| KK17        | Chroesthes longifolia (Wight) B. Hansen | 4 2                              | 6     |
| KK18        | Ancistroclados tecktorius (Lour.) Merr. | 3 2                              | 5     |
| KK19        | Ardisia colorata Wall. Ex Roxb.   | 5 4 1                           | 14    |
| KK20        | Dendropanax laurifolius (E. March.) Dcne. & Planch. ex R. C. Schneid. | 2 2 1 | 5 |
| KK21        | Zingerberaceae sp.                | 2                              | 2     |
| KK22        | Clerodendrum deflexum Wall.       | 3                              | 5 1   |
| KK23        | Cleistanthus sp.                  | 3                              | 1     |
| KK24        | Koompasia excelsa (Becc.) Taub    | 5                              | 2 7   |
| KK26        | Anacolosa frutescens (Blume) Blume | 4 3                              | 7     |
| KK27        | Justicia sp.                      | 3                              | 4 7   |
| KK28        | Psychotria condensa King & Gamble | 3                              | 2 5   |
| KK29        | Costus speciosus (J. Konig) Sm    | 3 2 2 3 10                  |       |
| KK30        | Zingerberaceae sp.                | 3                              | 1 2 8 |
| KK31        | Brassaiopsis polyacantha (Wallic) Banerjee | 4 3                              | 7     |
| KK32        | Eurycoma longifolia Jack          | 5                              | 1 6   |
| KT33        | Leptonychica caudata (Wall. ex G.Don) Burrett | 3 1 1                           | 5     |
| KT34        | Araceae sp.                       | 4                              | 2 2 8 |
| KT35        | Dioscorea hispida Denstn.         | 2                              | 3     |
| KT36        | Phyllanthus pulcher Wall. ex Mull. Arg. | 3 1 1                           | 5     |
| KT37        | Mimosa sp.                        | 3                              | 2 1 6 |
| KT38        | Thottea sp.                       | 4                              | 4 3 11|
| KT39        | Molineria latifolia (Dryand. ex W.T.Aiton) Herb. ex Kurz | 8 2 3 13                  |       |
| KT40        | Coeselipinia parviflora Prain     | 3                              | 3 6   |
| KT41        | Strychnos ignatii P. J. Bergius.  | 4                              | 1 5   |
| KT42        | Centotheca lappacea (L.) Desv.    | 2                              | 1 3   |
| KT43        | Zingerberaceae sp.                | 6                              | 4 3 13|
| KT44        | Rotheca serrata (L.) Steane & Mabb. | 4 4 2                           | 10    |

*KK - Kuala Keniam; KT - Kuala Trenggan
host the highest number of endophytes (14 isolates), followed by *Molineria latifolia* (13 isolates) and *Zingiberaceae* sp., KT43 (13 isolates).

Cytotoxicity of the extracts against P388 and K562 cell lines is shown in Table 2. Generally, the extracts were found to be more effective against P388 than the K562 cell line. Nearly half (47.6%) of the extracts showed activity (IC<sub>50</sub> of < 10 μg/ml) against P388 compared with 25% active against K562. These values were within the cutoff point of the National Cancer Institute criteria for cytotoxicity (IC<sub>50</sub> < 20 μg/ml) in the screening of crude plant extracts [8].

At IC<sub>50</sub> levels ≤ 1 μg/ml, 15.3% of extracts were active against P388 and 9.7% against K562 cell line. Very potent cytotoxicity (defined as IC<sub>50</sub> < 0.01 μg/ml) against P388 was shown by 3.3% of the extracts and 1.7% against K562. The ten endophytic extracts that showed very potent cytotoxic activity (IC<sub>50</sub> < 0.01 μg/ml) against P388 showed greater cytotoxicity than the pure compounds paecilosetin (IC<sub>50</sub> = 3.2 μg/ml) and farinosone (IC<sub>50</sub> = 1.1 μg/ml) isolated from an entomopathogenic fungi, *Paecilomyces farinosus* [9] and penicillenol (IC<sub>50</sub> = 2.6 μg/ml) from *Penicillium* sp. GQ-7, an endophytic fungi [10]. When compared with reported activity of compounds from marine organisms, 46 of the extracts (IC<sub>50</sub> < 1 μg/ml) showed greater potency than kulokekahilide-1, a cytotoxic depsipeptide from Chepalaspidean mollusk *Philinopsis speciosa* (IC<sub>50</sub> = 2.1 μg/ml) when tested against P388 [11]. The five extracts with IC<sub>50</sub> < 0.01 μg/ml against K562 were found to be more potent than the crude extract of *Aspergillus* sp. B-F-2 (IC<sub>50</sub> = 50 μg/ml) when tested against the same cell line [12]. However, these extracts were found to be less cytotoxic than chaetominine, a cytotoxic alkaloid produced by an endophyte *Chaetomium* sp. IFB-E015 which had an IC<sub>50</sub> of 0.008 μg/ml against K562 [13].

Only 24 isolates (8%) displayed antibacterial activity against at least one test microorganism with inhibition zones of 7 to 8 mm as shown in Table 3. Approximately

### Table 3: Antibacterial activity of extracts

| Endophytes | Antibacterial activity (mm) |
|------------|-----------------------------|
|            | Ec | Sa | Pa | Mi | Bs |
| KK1L2      | 7  | -  | -  | -  | -  |
| KK3R3      | 7  | -  | -  | -  | -  |
| KK4L1      | 7  | -  | -  | -  | -  |
| KK5L1      | 7  | -  | -  | -  | -  |
| KK5L4      | 7  | -  | -  | -  | -  |
| KK5R1      | 7  | -  | -  | -  | -  |
| KK6L3      | 7  | -  | -  | -  | -  |
| KK9L2      | 7  | -  | -  | -  | -  |
| KK9S3      | 7  | 7  | -  | -  | -  |
| KK9S5      | 7  | -  | 7  | -  | -  |
| KK9R1      | 7  | -  | -  | 7  | -  |
| KK11S2     | 7  | -  | -  | 7  | -  |
| KK12R2     | 7  | -  | 7  | -  | -  |
| KK16L1     | 7  | 8  | 7  | -  | -  |
| KK16L2     | 7  | -  | -  | 7  | -  |
| KK18S1     | 7  | -  | 7  | -  | -  |
| KK19S3     | 7  | -  | -  | -  | -  |
| KK27R1     | 7  | -  | -  | -  | -  |
| KK30S1     | 7  | -  | -  | -  | -  |
| KK30RH1    | 7  | -  | -  | -  | -  |
| KT33L1     | 7  | -  | -  | -  | -  |
| KT34L3     | 7  | -  | -  | -  | -  |
| KT34S1     | 7  | -  | -  | -  | -  |
| KT43L4     | 7  | -  | -  | -  | -  |
| Gentamycin  | 25 | 11 | 15 | 30 | 25 |

KK - Kuala Keniam; KT - Kuala Trenggan; L - Leaf; S - Stem; R - Root; RH - Rhizome Test microorganisms; Ec - Escherichia coli; Sa - Staphylococcus aureus; Pa - Pseudomonas aeruginosa; Mi - Micrococcus luteus; Bs - Bacillus subtilis. - : None detected.

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*Not able to obtain IC<sub>50</sub> after three independent tests.*
half of the active isolates displayed inhibitory activity against *E. coli*, however, none of the isolates were as potent as gentamicin sulphate. In contrast, other studies have shown that endophytes are a good source of antibacterial agents. Guimaraes et al. [16] screened extracts from 39 endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*, resulting in 5.1% active extracts against *S. aureus* and 25.6% active extracts against *E. coli*. An extract of *Streptomyces* sp. (SU9 06) isolated from the stem of a Malaysian plant was found to be as effective as oxacillin against *B. subtilis* [17]. Kakadumycin from *Streptomyces* sp. NRRL 30566 isolated from *Grevillea pteridifolia* was effective against *S. aureus* [18]. Munumbicin B and D that was isolated from *Streptomyces* sp. NRRL 30562, an endophytic fungus of *Kennedia nigriscans*, possessed antibacterial activity as effective as vancomycin against *S. aureus* [19].

**Conclusion**

In conclusion, this preliminary screening of rainforest fungal endophytes revealed their potential to yield potent bioactive compounds for drug discovery programmes. Extract KK29FL1, a *Sporothrix* sp., showed very potent cytotoxic effect indicating its possible potential for development as an anti-cancer drug and warrants further investigation.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

KR was the principal investigator who participated in the designing of the study, plant collection and writing of the manuscript. NAMNH participated in the plant collection, overall conduction of experiments and writing the manuscript. LSM and ALJC participated in the planning of the study, plant collection and writing the manuscript. IAW and ABAM participated in the planning of the study.

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**References**

1. Strobel GA, Daisy B: Bioprospecting for microbial endophytes and their natural products. *Microbial Mol Biol Rev* 2003, 67:491-502.
2. Strobel GA: Endophytes as sources of bioactive products. *Microbes Infect* 2003, 5:535-544.
3. Bacon CW, White JF, Stone JK: An overview of endophytic microbes: endophytism defined. In *Microbial endophytes* 1st edition. Edited by: Bacon CW, White JF. New York: Marcel Dekker, Inc; 2000:3-29.
4. Strobel GA, Yang XS, Sears J, Kramer R, Sidhu RS, Hess WM: Taxol from *Pestalotiopsis microspera*, an endophytic fungus of *Taxus wallachiana*. *Microbiology* 1996, 142:435-440.
5. Lang G, Blunt JW, Cummings NJ, Cole AJL, Munro MHG: Hirustide, a cyclic tetrapeptide from a spider-derived entomopathogenic fungus, *Hirsutella* sp. *J Nat Prod* 2005, 68:1303-1305.
6. Mosemann F: Rapid cationic assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J Immunol Methods* 1983, 65:55-63.
7. NCCLS - National Committee for Clinical Laboratory Standards: Performance standards for antimicrobial disc susceptibility tests. 8th edition, Approved Standard, document M2-A8, NCCLS, Wayne, Pennsylvania; 2003.
8. Lee CC, Houghton P: Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. *J Ethnopharmacol* 2005, 100:237-243.
9. Lang G, Blunt JW, Cummings NJ, Cole AJL, Munro MHG: Paecilomycin: a new bioactive fungal metabolite from a New Zealand isolate of *Paecilomyces farinaceus*. *J Nat Prod* 2005, 68:10-211.
10. Lin ZJ, Lu ZY, Zhu TJ, Fang YC, Gu QQ, Zhu WM: Penicillicins from *Penicillium* sp. GQ-7, an endophytic fungus associated with *Aegiceras corniculatum*. *Chem Pharm Bull* 2008, 56:217-221.
11. Kimura J, Takada Y, Inayoshi T, Nakao Y, Goetz G, Yoshida WY, Scheuer PJ: *Kulokekahihide-I*, a cytotoxic depsipeptide from the *Cephalaspidean Mollusk* *Philinus speciosus*. *J Org Chem* 2002, 67:1760-1767.
12. Liu R, Zhu W, Zhang Y, Tianjiao Z, Liu H, Fang Y, Gu Q: A new diphenyl ether from marine-derived fungus *Aspergillus* sp. B-74. *J Antibiot* 2006, 59:362-365.
13. Jiao RH, Xu S, Liu JY, Ge HM, Ding H, Xu C, Zhu HL, Tan RX: *Chaetomine*, a cytotoxic endophytic product from *Chaetomium* sp. IFB-E015. *Org Lett* 2006, 8:5709-5712.
14. Song YC, Li H, Ye YH, Shan CY, Yang YM, Tan RX: Endophytic naphthyrene metabolites are co-inhibitors of xanthine oxidase, *SW116* cell and some microbial growths. *FEMS Microbiol Lett* 2004, 241:67-72.
15. Zhan J, Burns AM, Liu MX, Faeth SH, Leslie GAA: Search for cell motility and angiogenesis inhibitors with potential antican- cer activity: beavericin and other constituents of two endophytic strains of *Fusarium oxysporum*. *J Nat Prod* 2007, 70:227-232.
16. Guimaraes DO, Borges WS, Kawano CY, Ribeiro PH, Goldman GH, Nomizo A, Thiemann OH, Oliva G, Lopes NP, Pupo MT: Biological activities from extracts of endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*. *FEMS Immunol Med Microbiol* 2008, 52:134-144.
17. Ghadin H, Zin HM, Sabaratnam V, Baday N, Basri DF, Lian HH, Sidik NM: Isolation and characterization of a novel endophytic *Streptomyces* SUK 06 with antimicrobial activity from Malaysian plant. *Asian J Plant Sci* 2008, 7:189-194.
18. Castillos U, Harper JK, Strobel GA, Sears J, Alaei K, Ford E, Lin J, Hunter M, Maranta M, Ge H, Yaver D, Jensen JB, Porter H, Robinson R, Millar D, Hess WM, Condon M, Teplow D: *Kakadumycins*, novel antibiotics from *Streptomyces* sp. *NRRL 3* an endophyte of *Grevillea pteridifolia*. *FEMS Microbiol Lett* 2005, 241:183-190.
19. Castillos UF, Strobel GA, Ford EJ, Hess WM, Conder H, Jensen JB, Albert H, Rosion R, Condon MA, Teplow DB, Stevens D, Yaver D: Munumbicins, wide-spectrum antibiotics produced by *Streptomyces* *NRRL 3* endophytic on *Kennedia nigriscans*. *Microbiol* 2005, 656, 148:2675-2685.

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