Biological Activity of Chemical Constituents
Isolated from Streptomyces sp. Tc052, an Endophyte in Alpinia galanga

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Abstract: Some endophytic actinomycetes (120) were isolated from the roots of Alpinia galanga. Identification of these endophytes was based on their morphology and amino acid composition of the whole-cell extract. Most isolates were classified as Streptomyces sp. (82), with the remainder belonging to Nocardia sp. (11), Microbispora sp. (3) and Micromonospora sp. (2). Eight isolates were unclassified and 14 were lost during subculture. The strain identified as endophytic Streptomyces sp. Tc052 strongly inhibited test microorganisms. This endophyte was cultured with organic solvent and the extract was purified on a column of silica gel to give a major component, which was identified to be kaempferol, isoscutellarin, umbelliferone and cichorinin on the basis of spectroscopic data. These compounds together with the extract were tested for their antimicrobial activity against bacteria and yeast using micro-dilution methods for the determination of Minimum Inhibitory Concentrations (MIC) and Minimum Micobactericidal Concentration (MMC). The MIC values obtained with the crude extract varied from 64-128 µg mL⁻¹ against tested microorganisms. All the isolated compounds showed various activities.

Key words: Alpinia galanga, biological activity, endophytic actinomycetes, kaempferol, isoscutellarin, umbelliferone, cichorinin

INTRODUCTION

Most plants are host to one or more endophytic microorganisms. By definition, these organisms live between the living cells of their respective host and cause no overt tissue damage. Usually, fungi are the most commonly isolated endophytic microorganisms, but recently the endophytic actinomycetes were isolated from the tissues of healthy plants (Shimizu et al., 2000, Castillo et al., 2002). Some endophytes produced antimicrobial agents that may be involved in a symbiotic association with a host plant (Ezra et al., 2002, Castillo et al., 2003). We have recently isolated endophytic actinomycetes from 36 plant species. The most prevalent group of isolates were the Streptomyces sp. occurring in 6.4% of the tissue samples of Zingiber officinale. Some of the isolates showed strong antifungal activity (Taechowisan et al., 2003). In a separate study 59 endophytic actinomycetes were isolated from the roots of Z. officinale and Alpinia galanga and tested against some phytopathogenic fungi. The strain identified as Streptomyces aureofaciens CMUAc130 showed the most effective antifungal activity (Taechowisan and Lumpyong, 2003). The major active ingredients from the culture filtrate were identified as 5,7-dimethoxy-4-p-methoxyphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin (Taechowisan et al., 2005). We report here the active constituent isolation from the roots of Alpinia galanga Swartz (Zingiberaeae) of another Streptomyces sp. Tc052. Extraction of the culture medium of Streptomyces sp. Tc052 afforded kaempferol, isoscutellarin, umbelliferone and cichorinin which displayed very strong antifungal and antibacterial activities.

MATERIALS AND METHODS

Isolation of endophytic actinomycetes: Five hundred samples of the root tissues of Alpinia galanga were collected from the environs of Nakorn Pathom, Thailand, during the period April 2005-March 2006. Most of them were healthy roots. The samples were washed in running tap water and cut into small pieces of ca. 4×4 mm². Tissue
pieces were rinsed in 0.1% Tween 20 for 30 sec, then in 1% sodium hypochlorite for 5 min and then washed in sterile distilled water for 5 min. Next the tissue pieces were surface sterilized in 70% ethanol for 5 min and air-dried in a laminar flow chamber. Finally the pieces were transferred to dishes of humic acid-vitamin (HV) agar (Oguro and et al., 2001) containing 100 μg mL⁻¹ nystatin and cycloheximide and incubated at 30°C for 1 month. The cultures were inoculated onto ISP-2 media (Shirling and Gottlieb, 1966) for purification and stock cultures.

**Antifungal activity of the actinomycetes isolates against fungi, yeast and bacteria:** The fungal pathogen Colletotrichum musae, the causative agent of anthracnose of banana (the representative of hypphal fungi of plant pathogen), was used for screening antifungal activity. This fungal pathogen was obtained from Dr. Wipaporn Phothi, Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand. It was grown on Potato Dextrose Agar (PDA). Mycelial disks of 8 mm diameter were cut from the pathogen colonies and transferred to the ISP-2 plates and positioned 6 cm away from each pre-grown actinomycete colony. For antagonistic action to Candida albicans ATCC90028 (the representative of budding yeast of human pathogen), the yeast was cultured in ISP-2 broth at 30°C for 24 h, the cells were diluted to 10⁵ cells mL⁻¹ in soft agar and then were overlayed on pre-grown actinomycete colonies on ISP-2 plates. For antibacterial activity, we used the solid media bioassay test against Staphylococcus aureus ATCC25923, Escherichia coli ATCC10536, Pseudomonas aeruginosa ATCC27853 and Bacillus subtilis ATCC6633. After incubation of the selected actinomycete strains for 7 days at 30°C on ISP-2 plates, an agar disk was recuperated and placed on nutrient agar plates covered by 3 mL of top agar containing 10⁵ cells mL⁻¹ of bacteria test strains. The plates were incubated at 30°C for 5-7 days (for C. musae) and for 24 h (for C. albicans and bacteria). The width of inhibition zones between the pathogen and the actinomycete isolates was measured.

**Identification of endophytic actinomycetes:** Isolated actinomycetes were observed for their morphological and biochemical characteristics. For morphological characteristics, presence of aerial mycelium, spore mass colour, distinctive reverse colony colour, diffusible pigment, sporophore and sporechain morphology were recorded after 10 days incubation on ISP-2 medium. Diaminopimelic acid isomers and sugars from whole-cell extract were analysed for chemotaxonomic studies (Becker et al., 1964; Boone and Pine, 1968).

**Extraction and purification of active compounds:** Among the 120 isolates of endophytic actinomycetes, the isolate Tc052 was found to be the best producer of antimicrobial substances. This isolate was selected for extraction and purification of the secondary metabolites. Spores of Streptomyces sp. Tc052 were used to inoculate 250 plates of ISP-2 and incubated for 14 days at 28°C. The culture medium was then cut into small pieces that were extracted with ethyl acetate (3×300 mL). This organic solvent was pooled and then taken to dryness under rotary evaporation to give a dark brown solid (5305 mg). The solid was separated by column chromatography using silica gel 60 (Merck, 0.040-0.063 mm) and CHCl₃:MeOH (20:1, 20:2, 20:3, 20:4 and 20:5) as the eluent to give active fractions, A-4 and A-5.

**Antimicrobial screening:** Quantitative antimicrobial screening was carried out using the disk diffusion assay as described in the protocols of the U.S. National Committee for Clinical Laboratory Standards (1997). A single colony of C. albicans and bacteria was cultured overnight in 1 mL Sabouraud broth (SB, for yeast) and nutrient broth (NB, for bacteria) at 37°C, after 12 h incubation, 0.5 mL of the culture suspension was added to 4.5 mL pre-weighed SB and the solution was incubated at 37°C to obtain cultures in the exponential phase of growth. The crude extract and purified compounds to be tested was dissolved in methanol (1 mg mL⁻¹) and 50 μL was applied to sterile (6 mm diameter) paper disks (Advantec, Toyo Roshi Kaisha, Ltd., Japan), dried and then placed on Sabouraud agar plate spreading with C. albicans or placed on nutrient agar plate spreading with test bacteria or placed on PDA plate, each plate was then incubated with an agar block (8 mm diameter) containing mycelial mats of the fungi in the center of the plate (the paper discs were 2.2 cm from the fungi). Incubation condition was 37°C for 24 h for bacteria and yeast and 30°C for 72 h for fungi. Results of the qualitative screening were recorded as the average diameter of the inhibition zone surrounding the paper disks containing the test substances and were reported. Each treatment consisted of three replicates. The experiment was repeated twice.

**Minimum Inhibitory Concentrations (MICs):** MICs of crude extract and purified compounds were determined by NCCLS microbroth dilution methods (National Committee for Clinical Laboratory Standards, 1997). The crude extract and purified compounds were dissolved in DMSO. A dilution suspension of bacteria and yeast was inoculated into each well of a 96-well microplate, each containing a different concentration of the test agents. We performed doubling dilutions of the test agents. The range of sample
RESULTS AND DISCUSSION

After 3-4 weeks incubation, the surface of some root tissue samples showed hyphal growth which subsequently grew out onto the surface of the HV agar (Fig. 1). Growth of bacteria and fungi from the root tissues was almost completely inhibited by the antibiotics included in HV agar leaving the actinomyceses clearly visible. The low level of bacterial contamination observed was due to Bacillus sp. This contamination may have arisen from spores on the surface of these tissues that were resistant to chemical surface sterilization or may be due to an endophytic Bacillus sp. (Bai et al., 2002). Incubation of surface-sterilized plant parts in a moist chamber and plating of plant tissues on agar media are techniques usually employed in plant pathology and not often used in microbial ecology. However, they may be extremely useful in the isolation of microorganisms from unusual habitats. Using these techniques, we were able to confirm the presence of endophytic actinomyceses in plant tissues, especially roots, where a large number of these organisms are most probably found. The actinomyces isolates took at least 3 weeks to grow cut from the tissues. If the tissue sterilization procedure used in this study was not sufficient to kill surface microbes, they would be expected to grow from specimens within a few days.

Five hundred samples of the root tissues of Alpinia galanga yielded at least 120 endophytic microorganisms. In total 120 isolates were recovered, the majority of which were Streptomycetes sp. (82), with the remainder identified as Nocardia sp. (11), Microbispora sp. (3), Micromonospora sp. (2). Eight isolates did not develop sporulating structures, although meso-diaminopimelic acid was detected in whole cell extracts, confirming an actinomyces status and 14 were lost during subculture. The antimicrobial activity of endophytic actinomyces isolates is shown in Table 1. The majority of the isolates (>50) appeared not to produce secondary metabolites which displayed antimicrobial
activity against all of the test microorganisms. The remaining isolates could be divided into five categories according to the size of the growth-inhibition zones produced. This survey revealed that only a small number was strongly inhibitory to test bacteria and fungi. In a similar study, Sardi et al. (1992) obtained ca. 509 isolates from the roots of 13 plant species and most of these were *Streptomyces* sp. They classified these isolates into 72 groups based on their characteristics. After testing antimicrobial activity of 10 groups against *Micrococcus luteus* and *Pseudomonas aeruginosa*, then found that all groups had antimicrobial activity against one or the other organisms, but not to both. Thus most of their isolates had a narrow antimicrobial spectrum. From the present study results of *in vitro* antimicrobial activity (Table 1), only two endophytic actinomycete isolates had a potential of antimicrobial activity to *S. aureus*, *E. coli*, *P. aeruginosa*, *S. subtilis*, *C. albicans* and *C. museae*. These results demonstrated that some of endophytic actinomycetes have the potential for inhibiting the growth of tested microorganisms.

An endophyte designated actinomycete Tc052 was of great interest, because of its potent antimicrobial activity. Morphological observation of 3-day-old cultures of Tc052 grown on ISP-2 medium revealed that sporesphores to be monopodially branched and fleshy, producing open spiral of oval-shaped spores (1×1.5 μm) with smooth surfaces (Fig. 2). The substrate mycelium was extensively branched with non-faceting hyphae. The aerial mycelium was white changing to ash-grey with yellow soluble pigment occasionally discernible. Based on results in morphological observation (light microscopy and scanning electron microscopy) as well as on the presence of LL-diaminopimelic acid in the whole-cell extract, endophytic actinomycete Tc052 was identified as belonging to the genus *Streptomyces*.

**Structure elucidation:** Purification of A-4 fraction using 16-20% MeOH in CHCl₃ afforded 37 mg of compound 1 and 18 mg of compound 2 and purification of A-5 fraction using 12-16% MeOH in CHCl₃ afforded 52 mg of compound 3 and 22 mg of compound 4.

**Compound 1:** Yellow crystals, m.p. 304-305°C. UV λmax(MeOH): 370, 266 nm (MeOH + NaOMe): 408, 277 nm, (MeOH + AlCl₃): 400, 278 nm, (MeOH + AlCl₃ + HCl): 400, 278 nm, (MeOH + NaOAc): 397, 277 nm. EIMS m/z (rel. abund. %): 286 (3) [M+, C₈H₇O₇], 256 (7), 128 (100), 118 (33), 113 (67), 97 (95). HNMR (D, CD₃OD): 7.09 (2H, d, J = 11.4 Hz, H-2', H-6'). 6.74 (2H, d, J = 11.4 Hz, H-3', H-4'). 5.95 (1H, d, J = 4.0 Hz, H-6), 5.14 (1H, d, J = 3.0 Hz, H-5). 13C NMR (D, CD₃OD): 144.3 (C-2), 136.8 (C-3), 170.9 (C-4), 164.4 (C-5), 99.9 (C-6), 160.9 (C-7), 92.3 (C-8), 149.5 (C-9), 107.8 (C-10), 125.3 (C-1'), 130.9 (C-2'), 116.1 (C-3'), 158.6 (C-4'), 116.1 (C-5'), 130.8 (C-6').

**Compound 2:** Dark yellow crystals, m.p. 300-301°C. UV λmax(MeOH): 282, 332 nm. Degradation occurs with all shift reagents. EIMS m/z (rel. abund. %): 286 (100) [M+, C₈H₇O₇], 258 (47), 257 (9), 168 (80), 140 (52), 118 (38), 112 (54). 1H NMR (D, CD₃OD): 7.06 (2H, d, J = 8.5 Hz, H-2', H-6'), 6.77 (2H, d, J = 8.5 Hz, H-3', H-5'), 6.08 (1H, s, H-3), 5.59 (1H, s, H-6).

**Compound 3:** Colorless needles, m.p. 228-229°C. It showed intense blue fluorescence under UV lamp and gave a negative Molisch's test. EIMS m/z (rel. abund. %): 162 (8) [M+, C₈H₇O₇], 149 (25), 138 (13), 121 (31), 110 (100), 94 (90), 81 (41), 66 (75), 55 (89). 1H NMR (D, CDCl₃ + CD₃OD): 7.59 (1H, d, J = 15.8 Hz, H-4), 7.07 (1H, br.s, H-8), 6.97 (1H, br.d, J = 8.1 Hz, H-6), 6.30 (1H, d, J = 7.8 Hz, H-3). 13C NMR (D, CDCl₃ + CD₃OD): 167.3 (C-2), 114.5 (C-3), 144.8 (C-4), 147.7 (C-4a), 114.7 (C-5), 121.2 (C-6), 144.2 (C-7), 113.4 (C-8), 145.0 (C-8a).

**Compound 4:** Transparent prisms, m.p. 215-216°C. It showed intense blue fluorescence under UV lamp and gave a positive Molisch's test. UV λmax(MeOH): 234, 289, 347 nm (MeOH + NaOMe): 249, 306, 390 nm, (MeOH + AlCl₃): 234, 289, 347 nm, (MeOH + AlCl₃ + HCl): 234, 289, 347 nm, (MeOH + NaOAc): 232, 282, 352 nm. EIMS m/z (rel. abund. %): 340 (2) [M+, C₈H₇O₇], 320 (2), 293 (72), 179 (90), 178 (97), 167 (90), 149 (100), 127 (88), 97 (56). 1H NMR (D, CDCl₃ + CD₃OD): 7.72 (1H, d, J = 9.5 Hz, H-4'), 7.11 (1H, s, H-6'), 6.94 (1H, s, H-5'), 6.19 (1H, d, J = 9.4 Hz, H-3'), 4.08 (1H, d, J = 7.3 Hz, H-1'). 13C NMR (D, CD₃OD): 3.31-3.85 (6H, H-2'-H-6').
NMR (δ, CDCl3 + CD3OD): 163.0 (C-2), 114.1 (C-3), 144.9 (C-4), 141.3 (C-4a), 113.3 (C-5), 148.9 (C-6), 149.8 (C-7), 104.7 (C-8), 149.99 (C-8a), 102.4 (C-1'), 76.0 (C-2'), 77.8 (C-3'), 74.0 (C-4'), 78.0 (C-5'), 61.7 (C-6').

Compounds 1, 2, 3 and 4 were identified as kaempferol (Markham et al., 1978), isoscutellarin (Jay and Gonnert, 1973), umbelliferone (Yamaguchi, 1970) and cichorin (Abdel-Salam et al., 1986), respectively by comparing their spectral data with those previously published (Fig. 3).

Results of the antimicrobial screening indicated that, the crude extract showed a wide range of activity, being effective against bacteria, yeast and fungi. Kaempferol and isoscutellarin showed strong activity against Gram positive bacteria (S. aureus and B. subtilis), while umbelliferone and cichorin showed moderate activity. Kaempferol and isoscutellarin exhibited moderate activity against Gram negative bacteria (E. coli and P. aeruginosa), while umbelliferone and cichorin showed weak activity. Kaempferol, isoscutellarin and umbelliferone showed strong activity against C. albicans and moderate activity against C. musae, while cichorin showed no activity against C. albicans and C. musae (Table 2). The antibacterial and antifungal activities of the crude extract and purified compounds were evaluated and the results are shown in Table 3. In general, there were differences in growth inhibition between compounds on various microbial cultures. The crude extract and purified compounds showed both antibacterial and antifungal activities at tested MIC and MMC limit of 256 μg mL⁻¹ (Table 3). The MIC values obtained with the crude extract varied from 64-128 μg mL⁻¹ to tested microorganisms.

For compounds isolated from the crude extract, the MIC values lower or equal to 128 μg mL⁻¹ were obtained with compounds 1, 2 and 3 on all tested microbial species (100%), compound 4 on 1 (16.66%) of the tested microbial species. Regarding the degree of activity of compounds isolated from the crude extract, the lowest MIC value (16 μg mL⁻¹) was noted with compound 1 on S. aureus and C. albicans, compound 2 on C. albicans. The results of the MMC determinations indicated that the MMC values lower or equal to 256 μg mL⁻¹ were observed with crude extracts on S. aureus and C. albicans (40%). Within this tested interval (0.5-256 μg mL⁻¹), the MMC values were obtained with compound 1 (100%), 2 (80%) and 3 (40%) of the tested microorganisms. The results of the MMC determinations indicated that cidal effect of many of the tested sample could be expected. However, a keen look of the results of MIC and MMC, showed that the MIC values obtained are two-three times lesser than MMCs on corresponding microorganisms, confirming the microbicidal effects of the concerned samples (Carbonnelle et al., 1987).

Previous reports indicated that kaempferol, isoscutellarin, umbelliferone and cichorin were produced by numerous species of plants, including Combretum erythrophyllum (Martini et al., 2004), Equisetum spp. (Milanovic et al., 2007), Scutellaria lateriflora (Gafner et al., 2003), Teucrium parvifolium, Trifora divaricata (Grauer et al., 2002), Arracaria toluensis (Figueroa et al., 2007) and Lomatia hirsuta (Erazo et al., 1997). The flowers of Impatiens balsamina contain kaempferol which are

Fig. 3: Chemical structures of kaempferol (1), isoscutellarin (2), umbelliferone (3) and cichorii (4)

Table 2: Antimicrobial activity of crude extract and purified compounds from Strophanthus sp. Tc052

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1: S.a.: Staphylococus aureus, E.C.: Escherichia coli, P.a.: Pseudomonas aeruginosa, B.s.: Bacillus subtilis, C.a.: Candida albicans, C.m.: Colletotrichum musae. 2: No activity, 1: (weak activity), 5-10 mm halo diameter, 2: (weak activity), 10.1-15 mm halo diameter, 3: (moderate activity), 15.1-20 mm halo diameter, 4: (strong activity), >20 mm halo diameter.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MMC) of crude extract and purified compounds

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known to possess antifungal, anticancer and antioxidant activities (Yang et al., 2001; Wang et al., 2006). Kaempferol was a markedly active inhibitor of transcriptional activation of COX-2 (Liang et al., 1999) and have inhibitory activity against melanin synthesis (Lim et al., 2006) and antibacterial activity against an acnes-inducing agent (Propionibacterium acne) with MIC values in the range <32-64 μg mL⁻¹ (Lim et al., 2007) and against Vibrio cholerae and Enterococcus faecalis, with MIC values in the range of 25-50 μg mL⁻¹ (Martini et al., 2004). While umbelliferone was weakly active against Mycobacterium fortuitum and Mycobacterium tuberculosis (Figueras et al., 2007). Present study is the first in which kaempferol, isocutellarin, umbelliferone and cichorin from culture of an endophytic Streptomyces species was isolated from the root tissue of Alpinia galanga and we confirm their antimicrobial activity against Staphylococcus aureus ATCC25932, Escherichia coli ATCC10536, Pseudomonas aeruginosa ATCC27853 Bacillus subtilis ATCC6633 Candida albicans ATCC50028 and Colletotrichum musae.

As stated in several reports, Streptomyces activity in plants not only protects against pathogens, but the metabolite products of Streptomyces also influence plant growth and physiology (Katznelson and Cole, 1965; Mishra et al., 1987). As kaempferol, isocutellarin, umbelliferone and cichorin has been isolated from endophytic Streptomyces sp. Tc052 and their antimicrobial activity was observed. These results indicated that some endophytic actinomycetes were potent for protection their host from phytopathogenic microorganisms.

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