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Antimicrobial Activity of Soil Fungi from Khao Nan National Park, Nakhon Si Thammarat Province, Thailand

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The main objective of this study was to evaluate antimicrobial activity of the soil fungi collected in Khao Nan National Park, Nakhon Si Thammarat Province, Thailand. The broth ethyl acetate extracts (BE) and the cell ethyl acetate extracts (CE) of soil fungi which displayed potential antimicrobial activity from primary screening were prepared and investigated for their antimicrobial activities against a number of human pathogens. Of 160 crude extracts, 81 crude extracts (50.63%) from 52 fungal species displayed antimicrobial activity against at least one of the tested strains. Indeed, the BE extracts was more effective than the CE extracts. MIC evaluation of these active extracts against each tested pathogen revealed that BE extract from *Trichoderma harzianum* WuHQ75 displayed a lowest MIC (1 μg/ml) against *Microsporum gypseum*; CE extract from *Aspergillus flavus* WuHQ25 exhibited the best MIC value at 16 μg/ml against *Staphylococcus aureus* ATCC25923; BE extract from *Trichoderma brevicompactum* WuLP33 and BE extract from *Trichoderma atroviride* WuGn24 showed the best MIC value at 16 μg/ml against *Candida albicans* ATCC90028; BE extracts of *Fusarium solani* WuLP66, unidentified sp.5 WuKL37, and *T. brevicompactum* WuLP33 displayed the best MIC value at 32 μg/ml against methicillin-resistant *S. aureus* SK1, *Escherichia coli* ATCC25922, and *Cryptococcus neoformans* ATCC90112, respectively. Among 81 extracts, only the CE extract from *Penicillium* sp. 2 WuGn15 exhibited MBC value at 128 μg/ml against methicillin-resistant *S. aureus* SK1. Overall, these results demonstrated that soil fungi are potential source for antimicrobial substances.

Key words: Antimicrobial activity, Soil fungi, Natural products.

With an increasing number of drug-resistant pathogens of humans, a concerted effort is needed to search for novel antimicrobial agents from diverse natural sources. Among them, fungi are chemically-rich source of secondary metabolites ranging from small molecules such as amino acid, peptides, polyketides, alkaloids and terpenes to large or complex molecules, e.g., peptides and enzymes[1]. It was reported that 22% of 12,000 antibiotics known in 1995 were produced by filamentous fungi[2,3]. Particularly, fungal-derived products, such as penicillins, cephalosporins, mevinolin, compactin, lovastatin, pravastatin and atorvastatin, are perhaps the best known

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antimicrobial agents. Furthermore, varieties of other compounds with distinctive pharmacological activities, such as fusarpyrones and beauvercin, have also been discovered as fungal metabolites[4,5,6,7].

Soil is regarded as a massive pool gathering several billion living organisms[8]. Among them, fungi are one of the dominant species found in soil[9]. It has been estimated that there are more than 75,000 species of soil fungi, which can be accounted for 5% of all fungi found in every part of the world[10]. Therefore, soil fungi have been focused as an alternative source to search for bioactive compounds[11]. Isaka *et al.*[12] reported a new bioactive metabolite produced by soil fungus, *Hamigera avellanea* BCC 17816 which inhibited the growth of bacteria. Numerous studies have showed that soil fungi are able to display antimicrobial activity[13,14,15,16,17].

Khao Nan National Park is a part of the Nakhon Si Thammarat mountain locating in southern part of Thailand. Indeed, it is one of the richest areas in terms of biodiversity[18]. The park is covered with tropically productive rain forests with high humidity, and continual rainfall brings about the cold weather year round. Due to its climatic pattern, Khao Nan National Park is an ideal place for the growth of soil fungi.

Therefore, the aims of the present study are to investigate the diversity of soil fungi in term of species composition in Khao Nan National Park and to evaluate their antimicrobial activities of the explored fungi. Screening of these soil fungi with antimicrobial activities against a number of human pathogenic microorganisms may provide the potential source for natural products with higher efficiency or novel ones.

MATERIALS AND METHODS

Isolation of soil fungi

Soil fungi were isolated from soils, which were collected from Khao Nan National Park, Nakhon Si Thammarat Province, Thailand. Two different methods were used to obtain fungal isolates in this study. Following the method described by Warcup[19], we performed the soil plate method in which 0.2-0.2 mg of well-pulverized soil from each sample was dispersed in a sterile Petri dish. Melted Glucose-ammonium nitrate agar (GAN) supplemented with 30 mg/l of streptomycin was poured over the soil, and then the plate was rotated gently to disperse the particles. For another method, the soil dilution plate method[20] was also performed. Ten grams of soil samples was suspended in 90 ml of normal saline solution and vortexed on a shaker. Dilutions of the suspensions (1:10, 1:100, and 1:1000) were prepared and 0.1 ml of these dilutions was eventually spread on GAN medium supplemented with 30 mg/l streptomycin. For both methods, the culture-plates were incubated at 26-28 ºC for five days. Different morphotypes of fungal isolates were subcultured on Malt extract agar (MEA). Lastly, the fungal isolates were identified based on conidial reproductive morphology and other reproductive structures.

Fungal cultivation and metabolite extraction

Each fungal isolate of different colonial morphotypes was grown on Potato dextrose agar (PDA) (Difco, USA) at 25ºC for 5-7 days. The agar culture was cut into 6 mm in diameter disc. Five pieces of agar culture were inoculated in 500 ml Erlenmeyer flasks containing 300 ml of Potato dextrose broth (PDB) (Difco, USA). All cultures were incubated at 25ºC for 3 weeks. After incubation, the fungal mycelia were separated from the culture medium by filtration. The 80 µl of filtrate was used for preliminary test of antimicrobial activity. Filtrates displaying antimicrobial activity in preliminary test were extracted three times with ethyl acetate and evaporated by a rotary evaporator under reduced pressure at 40-45 ºC to obtain the broth EtOAc extracts (BE). Fungal mycelium was soaked in a methanol solution for 2 days. The methanol extracts were re-extracted with ethyl acetate and evaporated by a rotary evaporator under reduced pressure at 40-45 ºC to obtain cell EtOAc extracts (CE).

Tested human pathogenic microorganisms

The soil fungi were investigated for their antimicrobial activities against seven human pathogens including *Staphylococcus aureus* ATCC25923, Methicillin-resistant *S. aureus* (MRSA) SK1, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC90028, *Cryptococcus neoformans* ATCC90112, and clinical isolates of *Microsporum gypseum* obtained from Songklanagarind Hospital, Prince of Songkla University.

Preliminary screening of antimicrobial activity with fungal culture broths

The antimicrobial activities of culture broths were preliminary tested by the agar well diffusion method[21]. Six mm in diameter wells were made by removing agar disc. The 80 µl of 3-week-old-culture broths was pipetted into each agar well. Activity was assessed using Mueller-Hinton agar(MHA) (Difco, USA) for bacteria and Sabouraud's dextrose agar (SDA) (Difco, USA) for yeast and fungi. Control antibiotic disc consisted of gentamicin (10 µg/disc) for *E. coli*, and *P. aeruginosa*, vancomycin (30 µg/disc) for *S. aureus*, amphotericin B (10 µg/disc) for yeasts and 30 µg of miconazole nitrate for dermatophyte (*M. gypseum*). Bacterial plates were incubated at 35 °C for 18-24 hours. Yeast plates were incubated at 35 °C for 18-24 hours for *C. albicans* and 48 hours for *C. neoformans* and fungal plates were incubated at 25°C for 7-10 days. Inhibition zones were measured in mm by a vernier caliper. All experiments were performed in triplicate.

Antimicrobial screening of fungal crude extracts

Antimicrobial activities of the soil fungal extracts (BE and CE) at a concentration of 200 µg/ml were evaluated by a modified CLSI broth microdilution method[22,23,24]. Briefly, DMSO was used to dilute the stock solutions of soil fungal extracts to 1:10. For the test of fungal extracts against pathogenic bacteria, Mueller-Hinton broth (MHB) (Difco, USA) was used as diluted solution, whereas RPMI-1640 was used to prepare the dilution for the test against pathogenic yeast and fungi. After the preparation of 1:10 diluted extracts, the further dilutions of 1:25 were performed to obtain concentrations of 400 µg/ml. Then, 50 µl of crude extract samples (400 µg/ml) was transferred to steriled 96-well microtiter plates for further step. To prepare the tested human pathogenic microorganism, the bacteria were prepared in normal saline solution (NSS) with an optical density equivalent to 0.5 McFarland. Then the dilution of bacteria suspension (1:200) in Mueller MHB was prepared to obtain the final concentration of 10^6 CFU/ml. In the case of pathogenic yeast strains, the initial optical density equivalent to 2 McFarland was prepared and diluted to 1:200 in Sabouraud's dextrose broth (SDB) (Difco, USA) to obtain a final concentration of 10^6 CFU/ml, while the initial spore-concentration of pathogenic fungal strains was adjusted to 4×10^3 - 5×10^4 CFU/ml in SDB. For evaluation of antimicrobial activity, 50 µl of crude extract and 50 µl of pathogen-suspension were sequentially added into the well to obtain a final concentration of crude extract of 200 µg/ml. Three replications of each test were performed. The mixtures were incubated at 35°C, 15 hours for bacteria, 35°C, 24 hours for *C. albicans*, 35°C, 48 hours for *C. neoformans*, and 25°C, 6 days for fungi. After incubation, the resazurin microtiter plate assay[25] was performed for antimicrobial activities of fungal crude extracts against bacteria and yeast. Thirty microliters of resazurin solution was added to each well. The mixtures then were re-incubated at the same temperatures, 3-5 hours for bacteria, 6 hours for *C. albicans* and *C. neoformans*, and 24 hours for fungi. The antimicrobial results were interpreted according to a change of resazurin color (blue). A change of color from blue to pink (resorufin) referred a negative result indicating the growth of microorganisms, whereas the remaining blue color referred a positive result indicating inhibition of the growth.

Standard antibiotics at a final concentration of 4 µg/ml were also included as positive control. Gentamicin was used as a control antibiotic for *E. coli* and *P. aeruginosa*. Vancomycin was used for inhibition of *S. aureus*. Amphotericin B was used against yeasts and Miconazole nitrate was used to inhibit *M. gypseum*. In addition, a mixture of 50 µl of microbial suspension and 50 µl of 2% DMSO was used as a negative control.

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the crude extracts from soil fungi

Fungal crude extracts displaying antimicrobial activity at concentrations of 200 µg/ml were subjected to MIC evaluation. Crude extracts were diluted by the serial two-fold dilution method from 128 to 0.25 µg/ml. The mixtures of crude extracts and suspension of tested pathogens were prepared and evaluated for antimicrobial activity according to previous step. Lowest concentrations of the extracts against each human pathogen were recorded as MIC values.

The MBC and the MFC of the soil fungal crude extracts were determined by a streaking

method. The mixture of crude extract and tested pathogen which displayed no pathogenic growth was streaked onto Nutrient agar plate (NA) (Difco, USA) for bacteria and SDA plate for yeasts and fungi. Plates were incubated under proper conditions described previously. No growth of tested pathogen in the cultured plates indicated bactericidal or fungicidal activity of the fungal extracts. Lowest concentrations of the extracts giving no visible growth of each tested pathogen on the cultured plates were recorded as MBC or MFC values.

Molecular identification of active soil fungi

Fungi exhibiting good antimicrobial activity were identified based on the analysis of the DNA sequences of the ITS1-5.8S-ITS2, ITS regions of their rRNA gene.

The DNA extraction was performed using CTAB method. The DNA sequence of fungal ITS region was amplified from chromosomal DNA using a pair of universal primers, ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')[26]. The PCR reaction were performed with the following cycles: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and final extension at 72 °C for 10 min[27]. The PCR fragments was analyzed on agarose gel electrophoresis and stained with ethidium bromide. The amplified products then were purified by the QIA quick PCR Purification Kit (QIAGEN). Amplified ITS1-2 fragments were cloned using pGEMT-Easy vectors (Promega Corporation, USA) and transformed into *Escherichia coli* strain TOP10F' (rk- *Rec*)[*SupE*(*lac- proAB*)*hsd rec AL*,*F* (*tra D36 laclqlacZ*)]. Ligation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones with the High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd, Taiwan). DNA of ITS region from each sample was sequenced by MACROGEN Company, Korea. Basic Local Alignment Search Tool (BLAST Search) was used to search for sequences of the closest match in the GenBank database.

RESULTS

Fungal isolation and antimicrobial activity screening

A total of 815 fungal isolates were recovered from soils obtained from Khao Nan National Park, Nakhon Si Thammarat Province. Among them, 282 soil fungal isolates with different colonial morphotypes were selected for primary screening of antimicrobial activity against seven human pathogens, including *S. aureus* ATCC25923, methicillin-resistant *S. aureus* SK1, *P. aeruginosa* ATCC27853, *E. coli* ATCC25922, *C. albicans* ATCC90028, *C. neoformans* ATCC90112, and *M. gypseum*. The culture broths of 282 fungal isolates were tested for antimicrobial activity by agar well diffusion methods. It was found that 80 of them (28.36%) showed positive antimicrobial activity against at least one of the tested microorganisms (data not shown). In fact, majority of positive isolates were found to be effective against one tested microorganism (51.25%). However, none of them was found to inhibit all tested microorganisms (figure 1A). According to the primary screening, we further performed crude extract preparation from the eighty positive fungal isolates. A total of 160 crude extracts, including 80 BE extracts and 80 CE extracts were obtained and further tested for antimicrobial activity using broth micro-dilution method against the same set of tested microorganisms. With the concentration of crude extract at 200 µg/ml, it was found that 81 crude extracts (50.63%) from 52 fungal species displayed antimicrobial activity against at least one of the tested pathogens. Fifty of them (31.25%) were BE extracts and 31 of the extracts (19.38%) were based on CE-type extraction (Figure 1B). In this study, antimicrobial activity of the crude extracts was more effective against *S. aureus* ATCC25923 and *M. gypseum* than other tested pathogens. By contrast, the crude extracts exhibited the least effectiveness of antimicrobial activity against *E. coli* ATCC25922 (Figure 1C). These results provide the evidence that soil fungi obtained from Khao Nan National Park are potential source for antimicrobial product discovery.

Evaluation of MIC, MBC or MFC

In the present study, 81 crude extracts displayed antimicrobial activity against the tested human pathogens in previous study were evaluated for MIC and MBC or MFC values ranging between 0.25-128µg/ml. The results are summarized in Table 1. Thirty-seven (45.67%) of crude extracts showed the antimicrobial activity at MIC values between 1-128 µg/ml against at least one of the tested microorganisms. Among them,

Table 1 MIC (µg/ml) and MBC or MFC (µg/ml) of 81 crude extracts from soil fungal isolates

Species[a]code No. (type of extracts)	Bacteria[b] MIC/MBC (µg/ml)			Fungi[c] MIC/MFC (µg/ml)			
	SA	MRSA	EC	PA	CA	CN	MG
1. *Aspergillus flavus*WuHQ25 (BE)	128/-[d]	-/-	-/-	-/-	-/-	-/-	-/-
2. *Aspergillus flavus* WuHQ25 (CE)	16/-	-/-	-/-	-/-	-/-	-/-	-/-
3. *Aspergillus flavus* WuHQ45 (BE)	64/-	-/-	-/-	-/-	-/-	-/-	-/-
4. *Aspergillus niger*WuLP03 (BE)	-/-	200/-	-/-	-/-	-/-	-/-	-/-
5. *Aspergillus niger* WuLP03 (CE)	200/-	200/-	-/-	-/-	-/-	-/-	-/-
6. *Aspergillus niger*WuLP26 (CE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
7. *Aspergillus niger*WuLP28 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
8. *Aspergillus niger*WuLP28 (CE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
9. *Aspergillus versicolor*WuGn59 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	32/-
10. *Aspergillus versicolor*WuGn59 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
11. *Aspergillus* sp.1 WuLP09 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	64/-
12. *Aspergillus* sp.1 WuLP09 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
13. *Aspergillus* sp.1 WuHQ48 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
14. *Aspergillus* sp.1 WuHQ48 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	128/-
15. *Aspergillus* sp.1 WuHQ49 (BE)	32/-	-/-	-/-	-/-	-/-	-/-	-/-
16. *Aspergillus* sp.1 WuHQ49 (CE)	32/-	-/-	-/-	-/-	-/-	-/-	-/-
17. *Aspergillus* sp.2 WuHQ06 (BE)	-/-	128/-	-/-	-/-	-/-	-/-	-/-
18. *Aspergillus* sp.2 WuHQ63 (BE)	64/-	-/-	-/-	-/-	-/-	-/-	-/-
19. *Aspergillus* sp.2 WuHQ63 (CE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
20. *Fusarium dimerum*WuKL41 (BE)	128/-	-/-	-/-	-/-	-/-	-/-	-/-
21. *Fusarium dimerum*WuKL41 (CE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
22. *Fusarium oxysporum*WuKL14(BE)	-/-	-/-	-/-	-/-	-/-	200/-	-/-
23. *Fusarium oxysporum*WuKL14 (CE)	-/-	-/-	-/-	-/-	200/-	-/-	-/-
24. *Fusarium solani*WuLP66 (BE)	64/-	32/-	-/-	-/-	-/-	-/-	-/-
25. *Fusarium solani*WuLP66 (CE)	-/-	64/-	-/-	-/-	-/-	-/-	-/-
26. *Fusarium* sp. WuGn71 (BE)	64/-	-/-	-/-	-/-	-/-	-/-	-/-
27. *Geotrichum* sp. WuGN54 (BE)	-/-	128/-	-/-	-/-	-/-	-/-	-/-
28. *Gliocladium virens*WuKL01 (BE)	-/-	200/-	-/-	-/-	-/-	-/-	-/-
29. *Gliocladium virens*WuLP48 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
30. *Mariannaea* sp. WuGn47 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
31. *Mariannaea* sp. WuGn47 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
32. *Metarhiziuman isophae*WuGn36 (CE)	-/-	-/-	-/-	-/	-/-	200/-	-/-
33. *Paecilomyces lilacinus*WuLP02 (BE)	-/-	-/-	-/-	-/-	128/-	-/-	-/-
34. *Paecilomyces* sp.3 WuLP24 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
35. *Penicillium* sp.2 WuGn15 (BE)	-/-	200/-	-/-	200/-	-/-	-/-	200/-
36. *Penicillium* sp.2 WuGn15 (CE)	128/-	64/128	-/-	200/-	-/-	-/-	200/-
37. *Penicillium* sp.4 WuKL15 (BE)	-/-	-/-	64/-	200/-	-/-	-/-	-/-
38. *Penicillium* sp.4 WuKL15(CE)	-/-	-/-	-/-	200/-	-/-	-/-	-/-
39. *Penicillium* sp.5 WuLP65 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
40. *Penicillium* sp.5 WuLP65 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
41. *Penicillium* sp.8 WuGn39 (BE)	-/-	-/-	128/-	-/-	-/-	-/-	-/-
42. *Penicillium* sp.14 WuGn72 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
43. *Penicillium* sp.14 WuGn72 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
44. *Trichoderma brevicompactum* WuKL46 (BE)	200/-	-/-	-/-	200/-	-/-	-/-	-/-
45. *Trichoderma brevicompactum* WuLP33 (BE)	-/-	-/-	-/-	-/-	16/-	32/-	-/-
46. *Trichoderma brevicompactum* WuLP37 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	128/-
47. *Trichoderma brevicompactum* WuLP37 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
48. *Trichoderma brevicompactum* WuGn70 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
49. *Trichoderma harzianum* WuHQ75 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	1/-

50. *Trichoderma harzianum* WuHQ75 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	32/-
51. *Trichoderma paraceramosum* WuLP49 (BE)	128/-	-/-	-/-	-/-	-/-	-/-	-/-
52. *Trichoderma pleurotum* WuLP23 (BE)	-/-	-/-	-/-	-/-	64/-	-/-	128/-
53. *Trichoderma pleurotum* WuLP23 (CE)	-/-	-/-	-/-	-/-	200/-	-/-	128/-
54. *Trichoderma pleurotum* WuLP63 (BE)	128/-	-/-	-/-	-/-	-/-	-/-	-/-
55. *Trichoderma pleurotum* WuLP63 (CE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
56. *Trichoderma atroviride* WuKL16 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	32/-
57. *Trichoderma atroviride* WuKL16 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	64/-
58. *Trichoderma atroviride* WuKL49 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
59. *Trichoderma atroviride* WuGn24 (BE)	32/-	64/-	-/-	-/-	16/-	64/-	-/-
60. *Trichoderma atroviride* WuGn24 (CE)	128/-	64/-	-/-	-/-	32/-	-/-	-/-
61. *Trichoderma atroviride* WuGn33 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
62. *Trichoderma atroviride* WuHQ57 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
63. *Trichoderma atroviride* WuHQ57 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
64. *Trichoderma atroviride* WuHQ61 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	128/-
65. *Trichoderma atroviride* WuHQ61 (CE)	-/-	-/-	-/-	-/-	-/-	200/-	200/-
66. *Trichoderma* sp.2 WuLP54 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
67. *Trichoderma* sp.4 WuLP56 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
68. *Trichoderma* sp.4 WuHQ58 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	200/-
69. *Trichoderma* sp.4 WuHQ58 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	64/-
70. *Trichoderma* sp.5 WuHQ23 (BE)	-/-	64/-	-/-	-/-	-/-	-/-	-/-
71. *Trichoderma* sp.6 WuLP08 (BE)	-/-	-/-	-/-	-/-	200/-	200/-	-/-
72. *Trichoderma* sp.6 WuLP08 (CE)	-/-	-/-	-/-	-/-	200/-	200/-	-/-
73. *Trichoderma* sp.7 WuGn08 (BE)	200/-	200/-	-/-	-/-	-/-	-/-	-/-
74.*Trichoderma* sp.8 WuKL66 (BE)	200/-	-/-	-/-	-/-	-/-	-/-	-/-
75. *Trichoderma* sp.11 WuLP62 (BE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
76. *Trichoderma* sp.11 WuLP62 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
77. *Trichoderma* sp.12 WuGn66 (BE)	128/-	-/-	-/-	-/-	-/-	-/-	200/-
78. *Trichoderma* sp.12 WuGn66 (CE)							
Sterile forms	200/-	-/-	-/-	-/-	-/-	-/-	200/-
79. Mycelia sterilia sp.9 WuGn50 (BE)	-/-	-/-	-/-	-/-	-/-	64/-	-/-
Unidentified species							
80. Unidentified sp.5 WuKL37 (BE)	64/-	64/-	32/-	200/-	-/-	-/-	128/-
81. Unidentified sp.5 WuKL37 (CE)	-/-	-/-	-/-	-/-	-/-	-/-	200/-
Standard antibiotic							
Vancomycin	1/NE[e]	2/NE					
Gentamicin			0.5/NE	2/NE			
Amphotericin B					0.5/NE	1/NE	
Miconazole							1/NE

[a] Fungal isolates were identified based on morphology of colony, conidia and conidiophore
[b] Tested human pathogenic bacteria : SA = *S. aureus* ATCC25923, MRSA= methicillin-resistant *S. aureus* SK1, EC= *E. coli* ATCC25922, PA= *P. aeruginosa* ATCC27853.
[c] Tested human pathogenic fungi : CA= *C. albicans* ATCC90028, CN= *C. neoformans* ATCC90112, and MG= *M. gypseum*
[d] - = No growth inhibition at the concentration tested

the lowest MIC (1 µg/ml) value of BE extract from *T. harzianum* WuHQ75 against *M. gypseum* was obtained in this study. Interestingly, it was equivalent to the MIC value of standard antifungal agent, miconazole (1 µg/ml). Based on types of the tested microorganisms, the best MIC value against *S. aureus* ATCC25923 was found to be 16 µg/ml of CE extract from *A. flavus* WuHQ25. Similarly, the MIC (16 µg/ml) values of BE extract from *T. brevicompactum* WuLP33 and of BE extract from *T. atroviride* WuGn24 were represented as the lowest values against *C. albicans* ATCC90028. In the case of methicillin-resistant *S. aureus* SK1, *E. coli* ATCC25922, and *C. neoformans* ATCC90112, the lowest MIC values against these pathogens were found to be 32 µg/ml from BE extracts of *F. solani* WuLP66, unidentified sp. 5 WuKL37, and *T. brevicompactum* WuLP33, respectively. However,

Table 2. A summary of molecular identification of active soil fungi, identified based on their genera and species and the similarity percentage found in GenBank databases provided by the Nation Center for Biotechnology Information (NCBI)

Fungal code	Fungal Genus/Species with the most related sequences	GenBank accession number of best hit	% similarity
WuHQ75	*Trichoderma harzianum*	KC139307	99
WuHQ25	*Aspergillus flavus*	FJ011539	99
WuLP33	*Trichoderma brevicompactum*	EU280087	100
WuGn24	*Trichoderma atroviride*	HM176575	100
WuLP66	*Fusarium solani*	KC254047	99
WuKL37	-[a]	-	-
WuGn15	*Penicillium* sp. F02	JF439496	99

[a]No ITS-region amplification product was obtained

none of the extracts displayed antimicrobial activity against *P. aeruginosa* ATCC27853 at MIC value ranging between 1-128µg/ml. Comparing to MIC values of standard antibiotics against *S. aureus*, methicillin-resistant *S. aureus* SK1, *E. coli* ATCC25922, *C. albicans* ATCC90028, and *C. neoformans* ATCC90112, the MIC values of the extracts against these pathogens were higher than those of standard antibiotics. Of all 81 extracts, it was found that only the CE extract from *Penicillium* sp.2 WuGn15 exhibited MBC of 128 µg/ml against methicillin-resistant *S. aureus* SK1 (Table 1).

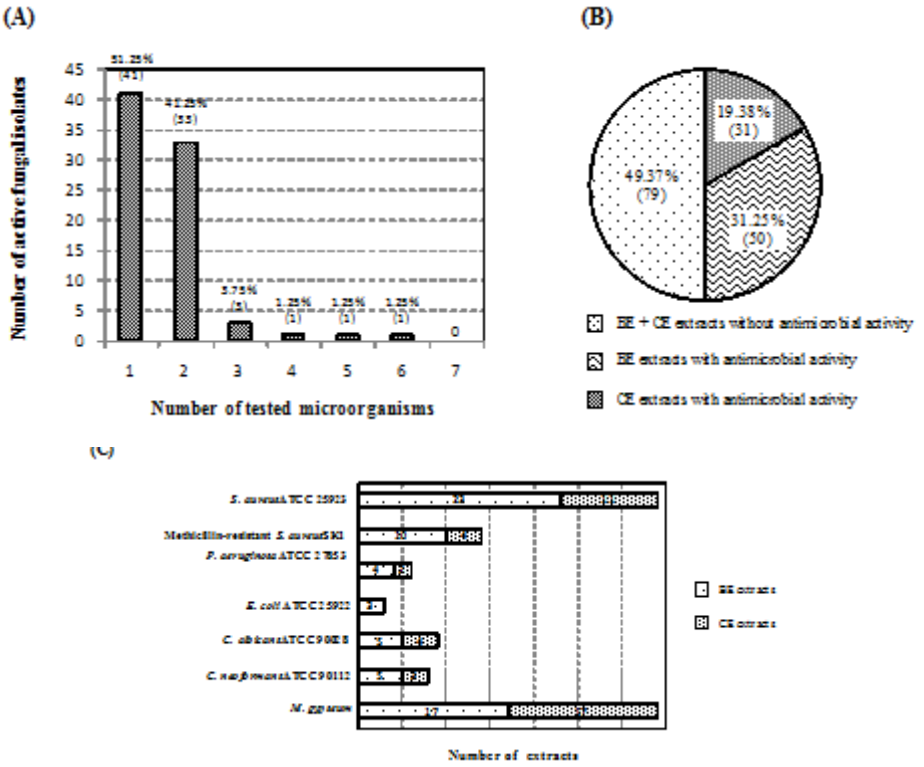

Fig. 1. Antimicrobial activity of soil fungal isolates and their crude extracts against human pathogenic microorganisms. (A) Number of active soil fungal isolates showed antimicrobial activities against number of tested microorganisms (B) Percentages of crude extracts with and without active antimicrobial activities (C) Number of active crude extracts with antimicrobial activities against each tested microorganism

Fungal species confirmation by molecular analysis

In this study, taxonomy of soil fungi was primarily based on colony morphology, conidial formation and conidiophore structure. To reconfirm species identification of the fungi which showed high antimicrobial activity, we analyzed the sequences of rDNA-ITS regions of these fungi. Six out of 7 fungal species could be identified through rDNA-ITS based approach, except for unidentified sp.5 WuKL37 due to lack of the product from rDNA-ITS region amplification. In fact, the fungal isolate WuKL37 has distinct morphological characteristics from other known fungi (data not shown). In this experiment, the sequence-blasting results reveal 99-100% identity with other published fungal ITS sequences in GenBank (Table 2). In addition, the ITS-DNA sequencing results were consistent with the morphological analysis for species identification.

DISCUSSION

The emergence of antibiotic and antifungal drug resistance of human pathogenic microorganisms remains a major public health problem worldwide. Consequently, this leads effort to discover and develop new active compounds to replace the defeated-ones. Over the past few decades, there has been growing interest in biodiversity research as it provides promising sources of valuable bioactive agents[28]. Distinct microbial habitats have been selected as sources for identification of active microbial species. This includes soil due to the fact that it is one of suitable habitats for the growth of microorganisms. Several research groups made their efforts to discover the active soil microbial species in hope of potential antimicrobial agent development[17,29,30]. Among them, fungi have been known as alternative sources for bioactive secondary metabolites[4].

In this study, the fungal species isolated from soil in tropical rainforest ecosystem, Khao Nan National Park were investigated for their antimicrobial activities against a number of human pathogenic microorganisms. As shown in figure 1A, most of culture broths from the active fungal species showed antimicrobial activity against one or two tested human pathogens. Similar result was also observed in MIC evaluation of the crude extracts (Table 1). Interestingly, within the range of concentrations tested in this study, the crude extracts which displayed antimicrobial activity against more than one tested microorganisms were most likely effective against a particular type of microorganisms, for example, a group of fungi but not bacteria or a gram positive bacteria, *S. aureus* but not others. These results may reveal a narrow spectrum of some antimicrobial substances which is favored for antibiotic therapy, since reduction of cost and toxicity of antimicrobial agents for therapeutic application can be achieved[31]. Among all active crude extracts, some crude extracts of *Penicillium* species and of *Trichoderma* species showed a broad spectrum of antimicrobial activity. In fact, *Penicillium* is described as "creative species" due to the fact that it produces varieties of active biomolecules[32], including antibacterial and antifungal metabolites[33,34]. Similarly, broad range of antimicrobial activities was observed in *Trichoderma* secondary metabolites. Isocyanide metabolite obtained from *Trichoderma hamatum* was shown to be effective against rumen bacteria[35], while biological activity of richotoxin peptaibols from *Trichoderma asperellum* was investigated against a gram positive bacteria, *Bacillus stearothermophillus*[36]. In addition, mycelial growth and sclerotial production of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* were shown to be affected by volatile metabolites from *Trichoderma viride*[37].

Comparison of the effectiveness between two extract types revealed that BE extract were more effective than CE extract against the same set of tested pathogenic microorganisms (figure 1B and table 1). Evidently, different profiling of extracellular and intracellular chemicals synthesized by fungi in the same culture condition was observed[38]. Indeed, primary metabolites such as triglycerides and fatty acids are recognized in intracellular extracts, whereas secondary metabolites tend to be secreted outside the cell[38]. In general, primary metabolites are related with fungal cellular function. For example, chemical components of fungal mycelia function as a source of energy[39]. In case of secondary metabolites, it has been shown to be involved in many aspects of fungal life processes, such as sporulation, sexual fruiting body formation[40,41], fungal pathogenicity[42], and fungal survival in restricted environment[43].

In our study, it was observed that gram

positive pathogenic bacteria were more sensitive to the crude extracts than gram negative bacteria as shown in figure 1C and table 1. The differential sensitivity could be due to different chemical constitutes of cell wall in each type of bacteria. Unlike gram positive bacteria, outer membrane of gram negative bacteria is rich of lipopolysaccharide (LPS) above a thin peptidoglycan layer. The LPS layer is impermeable to large molecules and hydrophobic compounds; therefore, it provides a protection to the organisms[44].

Evaluation of antimicrobial activity of fungal culture broths was able to identify more numbers of active species than that of fungal crude extracts. One of possible reasons is that some active constitutes may not be properly extracted due to unsuitable solvent extraction. It has been demonstrated that distinct profiles and proportions of volatile active compounds were obtained when culture filtrates of the fungus, *Monochaetia kansensis* were extracted with different solvents of varying polarities[45]. Therefore, the use of various solvents for crude extractions should be carried out in future studies

In the current study, we reported the fungal isolates exhibiting antimicrobial activity against some pathogenic microorganisms with MIC ranging from 1 to 200 µg/ml (Table 1). However, the most effective fungal isolates against each tested human pathogen except *P. aeruginosa* ATCC27853 displayed the MIC values within a range 1-32 µg/ml. As recommended in several studies, MIC value of crude extract below 100 µg/ml is considered as good antimicrobial activity[46,47]. Interestingly, *T. harzianum* WuHQ75 exhibited strong antimicrobial activity against *M. gypseum* with MIC value of 1 µg/ml comparable to that of miconazole, a standard antifungal drug. Therefore, these effective fungal isolates could be potent candidates for antifungal drug development. Several active species identified in the present study were in concordance with those reported by other research groups. For instance, antifungal and antibacterial activities of *T. harzianum* were reported to be effective against several microorganisms including fungi[48,49]. Cell wall-degrading enzyme such as protease (prb1) produced by *T. harzianum* was shown to have a role in biological control of *Rhizoctonia solani*[50]. In a previous study, the role of trichodermin, a toxin produced by *T. brevicompactum*, in antifungal activity against a number of yeasts including *C. albicans* was revealed[51]. In the case of *T. atroviride*, chitinase 42 (Chi42) was shown to inhibit mycelial growth of *R. solani*[52]. Furthermore, atroviridins A-C and neoatroviridins A-D, the peptaibols produced by *T. atroviride* were reported to be effective against gram positive bacteria and phytopathogenic fungi[53]. Antimicrobial activity of naphthoquinones and their derivatives from the Genus *Fusarium*, including *F. solani* and *F. oxysporum*, were evaluated. Some of these compounds displayed antibiotic activity against *S. aureus*[54]. The activity of antimicrobial compounds from *A. flavus* 092008, an endogenous fungus in Mangrove plant *Hibiscus tiliaceus* (Malvaceae) was investigated. It was mentioned that fermentative broth exhibited antimicrobial activity against MRSA *S. aureus* and *Enterobacter aerogenes*. Furthermore, an aflatoxinB_{2b} (1) compound from this fungus displayed moderate antibiotic activity against *Bacillus subtilis, E. coli,* and *E. aerogenes*[55]. Lastly, *Penicillium* species have long been exploited for human use as antibiotics. Their active compounds such alkaloids, diketopiperazines, quinolines, quinazolines, benzodiazepines, and polyketides were reported to play role in antimicrobial activity[32].

In this study, we were able to confirm the species of active fungi based on the molecular analysis of rDNA-ITS regions. However, PCR amplification of this region of the fungal isolate WuKL37 could not be achieved by using of the primers available in this study. Therefore, the attempt of molecular identification through rDNA-ITS sequences with other universal primer combinations should be carried out.

In summarization, our investigation suggests that soil fungal isolates from Khao Nan National Park, Nakhon Si Thammarat Province, Thailand are potential source to search for bioactive compounds. Several active soil fungal isolates reported in this study should be subjected to main active compound purification. The antimicrobial functions and structures of these promising purified active compounds should be elucidated in the near future.

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