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Antimicrobial activity of crude extracts prepared from fungal mycelia

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ABSTRACT

Objective: To evaluate the *in vitro* antimicrobial property of three different partitioned extracts (petroleum ether, ethanol and water) prepared from some fungal mycelia.
Methods: Seven fungal mycelia were prepared, initially extracted with acidified ethanol (0.2 mol/L HCl in 80% ethanol), yielding the raw crude extracts. The obtained extracts were then further partitioned with petroleum ether (F1), ethanol (F2) and water (F3). All the fractions were tested for antimicrobial activity using the disc diffusion assay.
Results: Our data showed that all the fractions could inhibit the testing bacteria. However, the inhibitory activity was found to be dependent on (i) the fungal strains used; (ii) the solvent extracted; and (iii) the testing bacteria assayed. In general, the ethanolic extracts (F2) derived from all fungi displayed highest inhibitory activity against the testing bacteria except for *Chaetomium* sp.
Conclusions: The findings of the present study concluded that the extracts prepared from the fungal mycelia had the bioactive compounds with antibacterial property. This study is a pioneering work and further study should be carried out for development of the new drug leads.

1. Introduction

Natural products derived from microbes are potentially useful in numerous fields which include agriculture, industry, and medicine [1]. Considered from the medical point of view, microbial metabolites have been the prime source of most antibiotics currently available on the market [2]. However, there is an alarming decrease in newly discovered antibiotics during the past decades. This is a consequence from many factors including the lack of interest from the industrial pharmaceutical company and the competition from the (semi) synthetic compounds as the source of drug leads. Such a situation eventually affects research and study of antibiotics screening which can be considered as an initial step in the pipeline of the pharmaceutical industry. This is not a good sign considered that there is an occurrence of multi-drug resistant organisms and newly identified pathogens [3].

Fungi represent an interesting group known to produce a wide range of bioactive compounds. Fungal metabolites are diverse in terms of structures and functions. In terms of biodiversity, fungi are possibly one of the most diverse species present on earth. It has been estimated that the fungal species number is between 1.5 and 5 million [4]. Recently, only a small proportion of the fungi (<100 000 species) has been described and it is thus of great interest to further explore the remaining fungal bioresources. In addition, the fungal metabolites are widely known to exhibit a broad range of biological properties including antimicrobial, antioxidant, and anticancer [5]. As a result, screening and isolation of metabolites from

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fungi are an interesting alternative and a challenge to reveal this untapped biological resource for finding the new bioactive compounds.

As a part of the research program focusing on the fungal diversity, we made an attempt to preliminary screen some fungal strains that were able to produce the metabolites that are active against microbial pathogens. The fungal isolates selected were based on (i) their taxa were known to synthesize the bioactive chemicals with biological activities; and (ii) some strains might represent new species (or strains). In addition, this study was one of the few work describing the antimicrobial activity of the fungal extracts derived from their mycelia.

2. Materials and methods

2.1. Fungal strains

Seven fungal strains used in this study were *Trichoderma* sp. DBM4197, *Aspergillus oryzae* DBM4336 (*A. oryzae*), *Botrytis cinerea* DBM4208 (*B. cinerea*), *Stachybotrys chartarum* (*S. chartarum*) DBM4297, *Fusicoccum aesculi* (*F. aesculi*) MFLU10-0260, *F. aesculi* MFLU10-0266, and *Chaetomium* sp. MFLU10-0761. The first four fungal strains were obtained from the Department of Biochemistry and Microbiology (DBM), University of Chemistry and Technology (Prague, Czech Republic), and the remaining three strains, isolated from dead leaves of *Magnolia liliifera* (*F. aesculi*) and *Cinnamomum iners* (*Chaetomium* sp.) were obtained from Mae Fah Luang University (MFLU).

2.2. Extraction and fractionation of fungal metabolites

All fungal isolates were cultured on Sabouraud dextrose agar for 30 days at 28 °C. After incubation, the fungal mycelia were scraped from the agar surface and used as the raw material for metabolite extraction. Initially, the mycelial samples (5 g) were mixed with 200 mL of 80% (v/v) ethanol containing 0.2 mol/L hydrochloric acid (HCl) with shaking at room temperature for 24 h. The solid materials were then pelleted by centrifugation and re-extracted with 200 mL of 80% (v/v) ethanol containing 0.2 mol/L HCl. Another extraction was performed with the solid materials using 200 mL of ethyl acetate. Subsequently, all the extracts obtained were combined, partially evaporated by rotary evaporator and then air dried to yield the fungal crude extracts (Figure 1). These crude extracts (0.5 g) were then partitioned using sequential extraction (with increasing polarity) with three different solvents (20 mL of each): petroleum ether (F1), ethanol

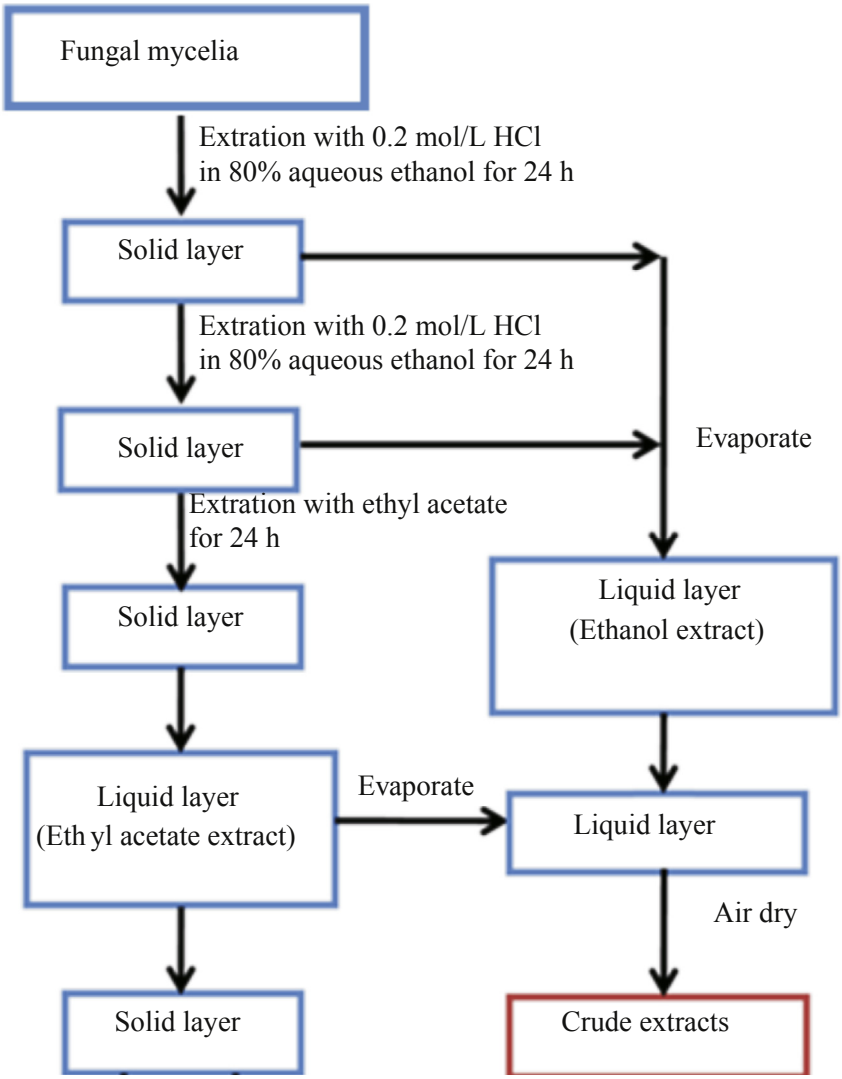

Figure 1. Overall procedure in preparation of crude extract from the fungal mycelia.

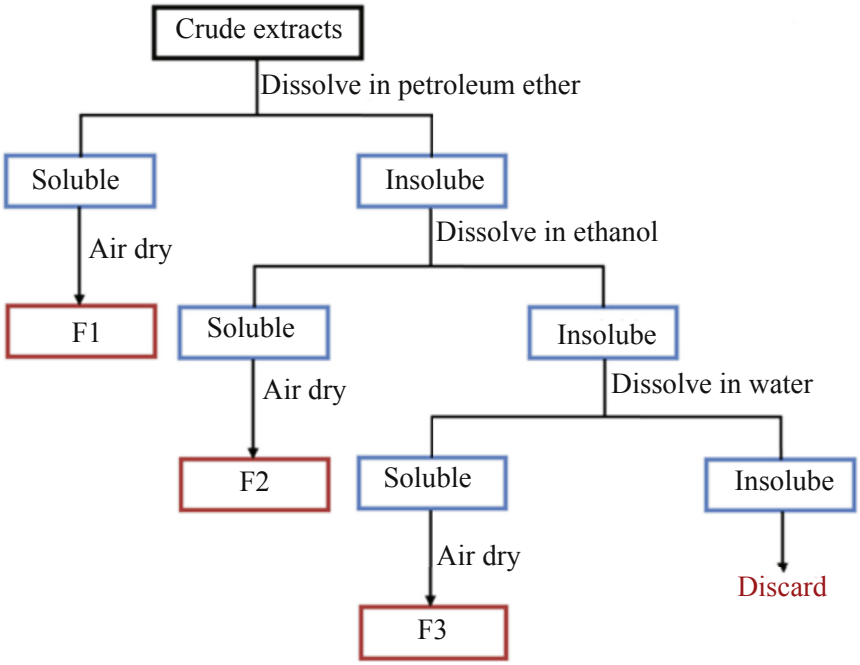

Figure 2. Sequential extraction of the dried crude extracts used in this study.

(F2) and water (F3) (Figure 2). All fractions were finally air-dried, weighed, and stored at −15 °C until further use.

2.3. Antimicrobial activity assay

The testing microbes used were five Gram-positive bacteria (*Bacillus cereus* DBM3035, *Enterococcus faecalis* DBM3075, *Micrococcus luteus* DBM3053, *Staphylococcus aureus* DBM3002, and *Staphylococcus epidermidis* DBM3072), two Gram-negative bacteria (*Enterobacter cloacae* DBM3126 and *Pseudomonas aeruginosa* DBM3081), and yeast *Saccharomyces cerevisiae* DBM2101. For microbial culture preparation, the testing bacteria were cultivated in nutrient broth at 37 °C for 18 h, whereas the yeast culture was cultivated in potato dextrose broth at 30 °C for 18 h. Then, the cell suspensions were adjusted to approximately 10^8 CFU/mL.

Antimicrobial activity assay was then examined by modified paper disc assay [6]. In brief, a freshly prepared culture of the testing microbes (10^8 CFU/mL) was inoculated on the surface of the nutrient agar plates (for bacteria), and the potato dextrose agar plates (for yeast). The sterile paper discs (6 mm in diameter) were then loaded with 20 μL of the fungal extract solutions (0.1 g/mL), air-dried thoroughly, and placed on the surface of the inoculated medium. These inoculated plates were then incubated for 24 h at 37 °C for bacteria, and 30 °C for yeasts. Each experiment was carried out in five replicates. The data, recorded by measuring the zone of growth inhibition around the discs (in mm), were expressed as means ± SD.

3. Results

3.1. Fungal strains and preparation of the fungal extracts

Seven fungal strains were selected to study their ability to produce the metabolites exhibiting inhibitory activity on some pathogenic microbes. The mass ratios of the three fractions obtained were recorded and presented as the relative data as shown in Table 1. It was found that the polar solvents appeared to be appropriate when using for preparation of the fungal metabolites as indicated by the high yields obtained. The ethanol solvent (F2) in particular resulted in the maximum yield of the extracts derived from *S. chartarum* DBM4297, *F. aesculi* MFLU10-0260, *F. aesculi* MFLU10-0266, and *Chaetomium* sp. MFLU10-0761; whereas the aqueous extraction (F3) gave highest yield of the extracts derived from *Trichoderma* sp. DBM4197, *A. oryzae*, and *B. cinerea*.

3.2. Antimicrobial activity

The result of the antimicrobial activity of the fungal mycelial extracts is presented in Table 1. It was found that all the fungal extracts (except the F3 extract of *B. cinerea*) tested were active and were able to inhibit at least one of the testing bacteria with different spectrum. It should be noted, however that all the extracts failed to suppress the growth of *S. cerevisiae*. Different solvents used for preparation of the mycelial extracts also gave different degrees in inhibitory activity. For this, the ethanol extracts (F2) prepared from most fungal mycelia were found to be more effective in inhibiting a wide range of the testing bacteria which included both Gram-positive and Gram-negative bacteria. For example, the F2 extracts derived from *A. oryzae*, *B. cinerea*, and *S. chartarum* DBM4297, were active against all seven testing bacteria used. Interestingly, the F1 and F3 extracts appeared to affect only the growth of the Gram-positive bacteria. Only one exception was the F3 extract derived from *Chaetomium* sp. MFLU10-0761 which showed inhibitory activity against the Gram-negative bacteria. Interestingly, two different strains of *F. aesculi* showed different results in antimicrobial activity, suggesting that the inhibitory property may also be strains-specific (or even substrains-specific).

Table 1
Mass ratio and antimicrobial activity of the crude extracts prepared from the fungal mycelia.

Fungus	Extract	Mass ratio	BC	EF	ML	SA	SE	EC	PA
Trichoderma sp. (DBM4197)	F1	0.21	6.0 ± 2.0	0.0 ± 0.0	4.0 ± 1.3	0.0 ± 0.0	1.0 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
	F2	0.33	5.0 ± 2.4	0.0 ± 0.0	12.0 ± 4.0	0.0 ± 0.0	2.0 ± 1.1	7.0 ± 2.2	6.0 ± 2.4
	F3	0.46	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A. oryzae	F1	0.12	20.0 ± 4.0	3.0 ± 0.7	3.0 ± 1.1	13.0 ± 5.0	2.0 ± 1.1	0.0 ± 0.0	0.0 ± 0.0
	F2	0.44	10.0 ± 2.0	1.0 ± 0.7	1.0 ± 0.4	11.0 ± 3.0	7.0 ± 3.1	1.0 ± 0.9	1.0 ± 0.4
	F3	0.44	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
B. cinerea	F1	0.08	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F2	0.14	8.0 ± 3.1	6.0 ± 2.7	5.0 ± 3.1	5.0 ± 1.8	6.0 ± 2.4	5.0 ± 1.6	5.0 ± 2.2
	F3	0.78	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S. chartarum (DBM4297)	F1	0.18	0.0 ± 0.0	3.0 ± 1.1	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F2	0.80	6.0 ± 1.8	5.0 ± 2.7	3.0 ± 1.7	6.0 ± 2.5	2.0 ± 0.9	7.0 ± 4.0	6.0 ± 2.4
	F3	0.02	10.0 ± 3.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
F. aesculi (MFLU10-0260)	F1	0.07	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.4	0.0 ± 0.0	1.0 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
	F2	0.73	6.0 ± 2.2	0.0 ± 0.0	5.0 ± 2.0	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 3.6	9.0 ± 3.3
	F3	0.21	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
F. aesculi (MFLU10-0266)	F1	0.11	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F2	0.75	7.0 ± 3.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 1.8	0.0 ± 0.0
	F3	0.14	4.0 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Chaetomium sp. (MFLU10-0761)	F1	0.09	7.0 ± 3.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F2	0.66	6.0 ± 3.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	F3	0.25	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 3.1	1.0 ± 0.4	2.0 ± 0.7

Mass ratio is presented as relative data. Antimicrobial activity is presented as means ± SD of five repetitions of the measurements of inhibition zones (mm). BC: *Bacillus cereus* DBM3035; EF: *Enterococcus faecalis* DBM3075; ML: *Micrococcus luteus* DBM3053; SA: *Staphylococcus aureus* DBM3002; SE: *Staphylococcus epidermidis* DBM3072; EC: *Enterobacter cloacae* DBM3126; and PA: *Pseudomonas aeruginosa* DBM3081.

4. Discussion

Fungal metabolites are diverse and exhibit various biological activities [7]. The results obtained from the present study further confirm this with an emphasis on antibacterial activity. The biodiversity of the fungi remains unclear but it is expected that the total species number can be up to 5 million [3]. It is therefore not a surprise to conclude that the fungi are and will remain one of the significant bioresources of the antibiotic discovery.

The *in vitro* trials showed that the selected fungal strains produced the metabolites exhibiting antibacterial activity albeit at varying degrees depending on the producing strain, extracting solvents, and the testing microbes. The fungi selected in this study are common saprobes and some are widely known to produce active extracellular metabolites with antimicrobial activity. For example, *Trichoderma* species secrete a plethora of metabolites and are presently used industrially as biocontrol agents [8]. *Aspergillus* species are also known to produce various bioactive compounds including mycotoxins [9,10]. Besides, some novel compounds with potential use in medicine are isolated and described from these fungi; these include atranones from *S. chartarum* [11], and chaetochromones from *Chaetomium indicum* [12].

It should also be noted however, that this is one of the few work dealing with the fungal mycelial extracts. Generally, many researchers have paid their interests on the extracellular metabolites as a consequence of many reasons. Some advantages include (i) the fungal cells remain viable; (ii) the metabolite's extraction is simple and easy; and (iii) large-scale production is convenient. However, it has been shown that the extract obtained from the fungal cells (*i.e.*, intracellular substances) also exhibited the biological activity. In some cases, the extracts obtained from the fungal mycelia displayed stronger activity [13,14].

To the authors' knowledge, this study is one of the few work describing the antimicrobial activity of the fungal extracts derived from their mycelia. Our results further confirm the potential use of the fungal metabolites in medicine and pharmaceutical industry. It is expected that the data obtained have opened up a new perspective in medical research to focus on the importance of a preliminary screening study, which is in fact a key step in the pharmaceutical pipeline research.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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References

[1] Demain AL. Importance of microbial natural products and the need to revitalize their discovery. *J Ind Microbiol Biotechnol* 2014; **41**: 185-201.
[2] Imhoff JF, Labes A, Wiese J. Bio-mining the microbial treasures of the ocean: new natural products. *Biotechnol Adv* 2011; **29**: 468-82.
[3] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. *P T* 2015; **40**: 277-83.
[4] Blackwell M. The fungi: 1, 2, 3 … 5.1 million species? *Am J Bot* 2011; **98**: 426-38.
[5] Kaul S, Gupta S, Ahmed M, Dhar MK. Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. *Phytochem Rev* 2012; **11**: 487-505.
[6] Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial disk susceptibility tests; approved standard-7th ed. CLSI document M02-A11*. Wayne: Clinical and Laboratory Standard Institute; 2012.

[7] Pelaez F. Biological activities of fungal metabolites. In: An Z, editor. *Handbook of industrial mycology*. New York: Marcel Dekker; 2005, p. 49-92.

[8] Zeilinger S, Gruber S, Bansal R, Mukherjee PK. Secondary metabolism in *Trichoderma* – chemistry meets genomics. *Fungal Biol Rev* 2016; **30**: 74-90.

[9] Yu J. Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. *Toxins (Basel)* 2012; **4**: 1024-57.

[10] Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal Bioanal Chem* 2009; **395**: 1225-42.

[11] Hinkley SF, Mazzola EP, Fettinger JC, Lam YF, Jarvis BB. Atranones A-G, from the toxigenic mold *Stachybotrys chartarum*. *Phytochemistry* 2000; **55**: 663-73.

[12] Lu K, Zhang Y, Li L, Wang X, Ding G. Chaetochromones A and B, two new polyketides from the fungus *Chaetomium indicum* (CBS.860.68). *Molecules* 2013; **18**: 10944-52.

[13] Meenupriya J, Thangaraj M. Isolation and molecular characterization of bioactive secondary metabolites from *Callyspongia* spp. associated fungi. *Asian Pac J Trop Med* 2010; **3**: 738-40.

[14] Meng LZ, Lin BQ, Wang B, Feng K, Wang LY, Cheony KL, et al. Mycelia extracts of fungal strains isolated from *Cordyceps sinensis* differently enhance the function of RAW 264.7 macrophages. *J Ethnopharmacol* 2013; **148**: 818-25.