Abstract:

The purpose of this research was to study antibacterial activity of crude extracts derived from the culture broths of two edible mushrooms, *Pleurotus citrinopileatus* and *Tricholoma crassum* Berk, cultivated in submerged fermentation. Potato dextrose broth (PDB) and yeast extract sucrose broth (YES) were used as culture media. Each culture filtrate was extracted and evaporated to yield a crude extract. Antibacterial activities against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were determined by paper disc diffusion assay. Crude extract (1 mg) of *P. citrinopileatus* culturing PDB showed the widest inhibition zones against *E. coli* ATCC 25922 (34.47 ± 0.50 mm) and *S. aureus* ATCC 25923 (33.73 ± 0.23 mm). Minimum inhibitory concentrations of this active crude extract against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, as determined by the broth microdilution method, were 5.0 mg/ml and 2.5 mg/ml, respectively. Crude extract of *T. crassum* culturing PDB showed weak activity against *S. aureus* ATCC 25923. YES broth was found to be inapplicable for cultivation of both mushrooms to produce antibacterial metabolite.

**Keywords:** *Pleurotus citrinopileatus*; *Tricholoma crassum* Berk.; Edible mushrooms; Crude extract; Antibacterial activity
Introduction

The increased occurrence of drug-resistant pathogenic bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* and *Streptococcus* sp. has prompted an urgent need to search for more and better antibiotics [1]. Microbes, especially fungi have been known to be a major source of bioactive compounds, for instance, *Penicillium chrysogenum* (penicillin), *Cephalosporium acremonium* (cephalosporin), *Penicillium griseofulvum* (griseofulvin), *Monascus ruber* and *Aspergillus terreus* (lovastatin) [2]. Mushrooms are a nutritionally functional food and a rich source of biologically active compounds including antimicrobial, antioxidant and anticancer substances [3]. Edible mushrooms in the genera of *Lentinula*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus* and *Tremella* have been reported to possess medicinal properties [4]. Crude extracts derived from some Portuguese wild mushrooms including *Cantharellus cibarius*, *Hypholoma fasciculare*, and *Ramaria botrytis* showed antibacterial activity against Gram-positive bacteria [5]. Two triterpenes, trichomycins A and B, separated from *Tricholoma* sp. AU-1 exhibited antibacterial activity against *Staphylococcus aureus* and *Streptococcus pneumoniae* and cytotoxic activity against a human leukemic cell line (THP-1) [6]. In addition, trichomalaides A-C (diterpenes) from *Tricholoma* sp. could induce neurite outgrowth in rat pheochromacytoma cells (PC-12) at a concentration of 100 µM [7]. Recently, a glucosylceramide isolated from *Pleurotus citrinopileatus* was found to be active against *Escherichia coli* and *S. aureus* with IC₅₀ values of 275.1 µM and 323.2 µM, respectively [8]. This background data prompted us to cultivate two edible mushrooms (*P. citrinopileatus* and *T. crassum*) in submerged fermentation and study antibacterial activity of crude extracts derived from the culture broths.

Materials and Methods

Sample preparation

Samples of the edible mushrooms, *P. citrinopileatus* and *T. crassum* were purchased from Biotechnology Research and Development Office, Department of Agriculture, Thailand. They were cultivated on potato dextrose agar (PDA) plate at 30°C for 7 days. Six pieces (6 × 6 mm²) of the grown culture were transferred into the 1,000-ml Erlenmeyer flask containing 200 ml of potato dextrose broth (PDB) and yeast extract sucrose (YES) broth at room temperature in stationary condition for 24 days.

Extraction of mushrooms

The culture broth was passed through four layers of cheese cloth, and then extracted with an equal volume of ethyl acetate (EtOAc) for 3 times. The organic layers were combined and evaporated at 40°C to give a crude extract. Four crude extracts obtained from this study including two crude extracts of *P. citrinopileatus* culturing PDB (181 mg) and YES (598 mg), and two crude extracts of *T. crassum* culturing PDB (530 mg) and YES (2,810 mg).

Determination of antibacterial activity

Preliminary screening for antibacterial activity

Antibacterial activity of four crude extracts was determined by paper disc diffusion assay. The tested bacteria were *Escherichia coli* ATCC 25922 (Gram-negative bacteria) and *Staphylococcus aureus* ATCC 25923 (Gram-positive bacteria) provided from Faculty of Agriculture and Life science of Chandrakasem Rajabhat University. Each bacterial strain was grown in tryptic soy broth at 37°C for 2-3 h. and suspended in 0.85% NaCl. The turbidity of bacterial suspensions was adjusted to match that of 0.5 McFarland standard (OD 0.1 at 625 nm). A sterile cotton swab was used to apply each bacterial suspension onto the entire surface of the Mueller Hinton agar plate. Three sterile blank disks (6 mm diameter) and ampicillin disc (BD™) were distributed evenly onto the inoculated agar surface. Ten µl of 100 mg/ml of crude extracts in dimethyl sulfoxide (DMSO) were applied to the blank discs yielding 1 mg/disc. DMSO was used as a negative control and ampicillin (10 µg/disc) was used as a positive control. After keeping at room temperature for 1 h, the test plates were incubated at 37°C for 24 h. These were done in triplicate. The results were the mean values of the inhibition zone diameters ± the standard deviations (S.D.).
Determination of minimum inhibitory concentration (MIC)

Antibacterial activity of the crude extracts against the two pathogenic bacteria was determined by broth microdilution method as described in the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) M7-A4 method [9]. Bacterial strains were grown on Mueller Hinton agar (MHA) for 24 h at 37°C. Selected fresh single colonies were inoculated into 5 ml of tryptic soy broth and incubated in shaking incubator for 2-3 h at 37°C. The turbidity of the bacterial suspension was adjusted with sterile normal saline solution to match the turbidity of 0.5 McFarland standard (OD 0.1 at 625 nm). Then the suspension was diluted with Mueller Hinton broth (MHB) to contain 1 Ó 10^6 CFU/ml. Solution of isolated compound in DMSO (25.6 mg/ml) was diluted with MHB for assays of antibacterial activity. The crude extracts were tested over final concentration range of 10 to 0.5 mg/ml. Oxacillin was used as positive control. MIC is defined as the lowest concentration that inhibits growth of test microorganism.

A 50-µl volume of MHB containing crude extract was dispensed into each well of sterile microtitre plates (96-flat-bottom wells). Sterile extract-free medium containing the corresponding amount of DMSO (25.6 mg/ml) was diluted with MHB for assays of antibacterial activity. The crude extracts were tested over final concentration range of 10 to 0.5 mg/ml. Oxacillin was used as positive control. MIC is defined as the lowest concentration that inhibits growth of test microorganism.

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By disc diffusion assay, 1 mg of the crude extract from P. citrinopileatus culturing PDB demonstrated strong activities against E. coli ATCC 25922 (higher than activity of 10 µg ampicillin) and S. aureus ATCC 25923 (less than activity of 10 µg ampicillin), as shown in Table 1. The crude extract from T. crassum culturing PDB showed weak and no activity against S. aureus ATCC 25923. YES broth was found to be inapplicable for promoting the production of antibacterial metabolite(s) by these mushroom (Table 1), in contrast to previous reports of other fungi [11]. This finding suggests that (1) the effect of culture medium is highly unpredictable and variable, which is in agreement with previous report by Wiyakrutta and colleagues [12], and (2) PDB used for submerged fermentation of P. citrinopileatus could induce the mushroom to secrete active metabolite(s) into the culture medium. Culture media have been known to affect the production of fungal secondary metabolites [11, 12]. For example, the endophytic fungus mitosporic Dothideomycete sp. LRUB20 have been reported to produce different metabolites when cultivated in malt Czapek medium and Czapek yeast autolyase medium [13, 14].

Based on primary screening, the crude extract with best activity was sequentially assayed for the minimum inhibitory concentration (MIC) values. The crude extract from P. citrinopileatus culturing PDB inhibited the growth of E. coli ATCC 25922 and S. aureus ATCC 25923 with MIC values of 5.0 mg/ml and 2.5 mg/ml, respectively, as shown in Table 2. This implied that
activities of the crude extract against Gram-negative bacteria and Gram-positive bacteria were comparable.

In conclusion, submerged fermentation of *P. citrinopileatus* in PDB could be used to cultivate the mushroom for the production of antibacterial metabolites. This alternative way instead of direct extraction from mushroom fruiting body might yield novel metabolites.

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