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Original Article

STRUCTURE CHARACTERIZATION AND EVALUATION POTENTIAL OF ANTIMICROBIAL EXTRACTS FROM *PHELLINUS LINTEUS* AGAINST SKIN INFECTIOUS PATHOGENS, *STAPHYLOCOCCUS EPIDERMIDIS* ATCC12228 AND *PROPIONIBACTERIUM ACNES* DMST14916

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ABSTRACT

Objective: The objective of this study was to investigate the antimicrobial activity of *Phellinus linteus* against skin infectious pathogens, *Staphylococcus epidermidis* ATCC12228 and *Propionibacterium acnes* DMST 14916.

Methods: Fungal fruiting bodies were extracted with 95% ethanol and ethyl acetate, and then, vaporized. The antimicrobial activities were determined by the disc diffusion method against *Propionibacterium acnes* DMST 14916 and *Staphylococcus epidermidis* ATCC12228 skin infectious pathogens. A minimum inhibitory concentration (MIC) and a minimum bactericidal concentration (MBC) for those crude extracts were determined. Finally, the chemical profile of crude extract was determined by using thin layer chromatography and GC-MS.

Results: The result demonstrated that the ethanolic extraction had more active fractions with an MIC of 0.5 mg/ml against the growth of *Propionibacterium acnes* DMST 14916 and *Staphylococcus epidermidis* ATCC12228 and also showed a minimum inhibitory concentration (MBC) at a concentration of 1.0 mg/ml, while ethyl acetate-based solvents failed to develop on TLC according to Retention factor (R_f) values of 0.71-0.76. The GC-MS was applied to investigate the chemical profile of crude extract *of Phellinus linteus*, revealing a component of hexadecanoic acid and 9, 12-octadecadienoic acid.

Conclusion: *Phellinus linteus* fruiting body extracts have great potential as antimicrobial compounds against *Propionibacterium acnes* DMST 14916 and *Staphylococcus epidermidis* ATCC12228. Thus, they can be used in the treatment of infectious diseases caused by bacterial pathogens.

Keywords: Antimicrobial activity, *Phellinus linteus*, *Staphylococcus epidermidis*, *Propionibacterium acnes,* MIC, MBC

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INTRODUCTION

In recent years, the increasing antimicrobial resistance called superbugs has driven a critical need to develop a novel antimicrobial agent [1]. The multi-drug resistant strains do not only occur through nosocomial infection, but also in the public condition [2] especially bacterial resistance in sexually transmitted infections [3], *Escherichia coli*, *Klebsiella pneumonia, Haemophilus* sp., vancomycin-resistant *Enterococcus* (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA), as well as opportunistic pathogens [4]. The main reason for the antibiotic-resistant crisis can be the overuse of broad-spectrum antibiotics. In addition, drugs used in livestock and agriculture badly affect the environmental microorganisms [5]. However, several novel antibiotic agents that are most suitable for use in the hospital environment have been developed to combat both Gram-positive and Gram-negative bacteria, especially screening active biomolecules with effective pharmacologic properties and drug safety with a return to treatment using traditional medicines.

Medicinal mushrooms in the class Basidiomycetes, *Phellinus linteus* (Berk. and M. A Curtis) Teng, called Krathin Phiman in Thai, have been widely used as herbs in China, Japan, Korea, and Thailand since the ancient time. *Phellinus linteus* has been found to produce several biologically active compounds that are usually associated with cell walls. These compounds have been suggested to contribute to the enhancement of immunity [6] and tumour-retarding effect, antioxidant [7-9], anti-inflammatory [10] and antimicrobial activities, [11] and good therapeutic effects on diabetes and obesity [12].

Hypothetically, the *Phellinus linteus* extract may be a reason that plays a significant role in the treatment of skin infectious pathogens. Thus, this study was conducted to analyze antimicrobial activities and investigate the chemical profile of crude extract compared with original positive drugs for an inhibition of microbial growth.

MATERIALS AND METHODS

Preparation of mushroom extract

The fruiting bodies of *Phellinus linteus* were obtained from a folk medicine company in Thailand. The extraction of the mushrooms was conducted by macerating the mushrooms in 95% ethanol and ethyl acetate at 1:20 (w/v) for 7 d. After the maceration, the extract was filtered through Whatman No. 1, and the filtrate was evaporated using an evaporator. The residue was extracted two times. Then, the extract was combined and evaporated to a constant weight. The crude extract was calculated for the percentage yield and kept at-20 °C for further investigation.

Preparation of inoculums

Both bacterial strains *S. epidermidis* ATCC12228 and *P. acnes* DMST 14916 were obtained from the National Institute of Health, Thailand. The tested bacteria were inoculated in nutrient broth and incubated for 16-18 h at 37 °C in aerobic condition for *S. epidermidis* ATCC12228 and in anaerobic condition for *P. acnes* DMST 14916. A 0.5 McFarland standard was used to adjust the turbidity corresponding to 10^7-10^8 CFU/ml.

Antimicrobial assay

The disc diffusion method was used to determine the antibacterial activity of the *P. linteus* extracts by using nutrient agar (NA). A fresh bacterial suspension with the turbidity corresponding to 10^7-10^8 CFU/ml was spread on NA plates with sterile cotton swabs.

The disc diffusion assay was completed by adding the tested crude extract which dissolved in dimethyl sulfoxide (DMSO) solution at an initial concentration of 100 mg/ml onto the 6 mm disc. The Petri plates were then incubated at 37 °C for 24 h in an incubator. The experiment was conducted in triplicate and the mean diameter of

the zone of inhibition was recorded in millimetres (mm). The results were represented by mean±standard deviation [13].

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

To assess the MIC and MBC of mushroom crude extracts, the serial two-flow dilution was carried out with the disc diffusion assay. The MIC represented the lowest concentration showing an inhibition zone; the MBC was determined by subculturing which showed no bacterial growth on the agar plates after incubated at 37 °C for 24 h in an incubator. The lowest concentration that did not show bacterial growth was defined as an MBC value. The experiment was conducted in triplicate, and the mean diameter of the zone of inhibition was recorded in millimetres (mm). Erythromycin was used as a positive control, and dimethyl sulfoxide (DMSO) solution was used as a negative one. All experiments were performed in triplicate. The results were represented by mean±standard deviation [14].

Thin layer chromatography analysis

TLC was carried out using aluminium silica gel 60GF254 of a thickness of 0.2 mm, (Germany). Standard chromatograms of *P. linteus* extract were prepared by applying a 20 µl extract solution to a silica gel TLC plate and developed with chloroform/methanol (1:1; v/v) under saturated conditions. The chromatograms were detected by UV-light (254 nm and 365 nm) and the colour reaction with a 5% sulfuric acid-ethanol spraying solution after heating at 100 ˚C.

Gas chromatography-mass spectrometry analysis

GC-MS analysis tests for the fruiting body extract composition analysis were performed on an Agilent 789 GC system instrument equipped with HP-5MS (5% diphenyl 95% dimethylpolysiloxane) column (30 m x 0.25 mm, 0.25 µm) and interfaced to a 5975C inert XL MSD with Triple-Axis Detector. A volume injection of 2 µl was employed (a split ratio of 10:1) at an injector temperature of 250 °C. The column temperature was increased from 60 °C to 250 °C at a rate of 5 °C/min. The outlet temperature was 280 °C. Mass spectra were taken at 70 Da. and Ms transfer line temperature of 250 °C. The components of the extract were identified by comparison of fragmentation patterns in mass spectra with those stored on the spectrometer database and reported in the literature. The relative percentage of the individual components was calculated from the GC peak areas [15].

Identification of bioactive constituents

Interpretation on Mass-spectrum GC-MS was carried out by using the database of National Institute Standard and Technology (NIST) containing more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST library. The name, molecular formula, weight and chemical structure of the components of the test materials were ascertained [16].

Statistical analysis

The results of the data experiments were expressed in mean±SEM (Standard Error of Mean) for groups (n=3).

RESULTS AND DISCUSSION

Antibacterial activities

The antimicrobial activity results of *Phellinus linteus* fruiting body extracts are summarized by the paper-disc diffusion method as shown in table 1. The results of the disc diffusion method in terms of the size of inhibition zone (mm) for the extracts were compared against microorganisms studied. The highest inhibitory activity was determined against *S. epidermidis* ATCC12228 and *P. acnes* DMST14916 in a clearing zone of 15.67±1.15 and 12.67±1.53 mm, whereas ethyl acetate extract showed no inhibition zone.

In a previous study, numerous *Phellinus* mushroom extract was reported as having an antimicrobial activity against Gram-positive bacteria, *B. cereus, B. subtilis* and *S. aureus* [17]. The mushroom in the fraction of methanol extract demonstrated a good activity against MRSA [18, 19]. In addition, the sesquiterpenoid, a bioactive compound from *Phellinus* species such as *P. fastuosus, P. merrillii, P. aureobruneus, P. crocatus, P. lloydii,* and *P. sublinteus* had an effective antibacterial activity against different microorganisms such as *Acinetobacter calcoacetic* NCIB2886, *B. subtilis* NCIM2010, *Candida albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* ATCC 2091, *Escherichia coli* MTCC 724, *E. coli* MTCC 739, *E. coli* ATCC 2046, *Klebsiella pneumonia* MTCC 432, *Proteus mirabilis* MTCC1429, *Pseudomonas aeruginosa* ATCC2036, and *S. aureus* HAL 2079, respectively [20].

Table 1: Bacterial activities of the crude extract of *Phellinus linteus*

Bacterial strains	Inhibition zone (mm.)			
	EtOH	EtOAC	+ve	-ne
S. epidermidis ATCC12228	15.33±0.58	-	22.67±0.58	-
P. acnes DMST14916	12.67±1.53	-	24.00±0.00	-

+ve: positive control (10.0 mg/ml erythromycin);-ne: negative control (10% DMSO); statistical analysis as mean±sd.

Bacteriostatic and bactericidal effect

In terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *S. epidermidis* ATCC12228 and *P. acnes* DMST14916, a lower MIC was observed in table 2. The MIC test indicated that the extract of the *P. linteus* fruiting body exhibited a minimal value of MIC (0.5 mg/ml) against *S. epidermidis*. MBC was found to have a concentration of 1.0 mg/ml with all strains. According to a previous study, *P. gilvus* aqueous extract showed MIC against *L. plantarum* ACC14917, *K. pneumonia* ATCC 10031 and *E. coli* ATCC25922 at concentrations of 45.0, 90.0 and 360.0 mg/l, respectively, while those of MBC against them were 90, 180, and 720 mg/l, respectively [21].

Chemical characterization of *Phellinus linteus*

Thin layer chromatography analysis

Developed TLC plates with visualized spots were observed. There were dark blue spots of extracts under 365 nm and 254 nm UV light ($R_f \approx 0.71$-0.76). After colourized by 5% sulfuric acid-ethanol solution, polar spots appeared in the same UV light positions.

Gas chromatography-mass spectrometry analysis

The active principles with their retention time (RT), molecular formula, molecular weight (MW), concentration (Peak area %) and the chemical structure were analyzed.

Table 3 shows the components present in the unfractionated ethanolic extract as identified by GC-MS. Two components comprising about 98.50% of the total ethanolic extract were identified with hexadecanoic acid, ethyl ester ($C_8H_{36}O_2$, 74.9%) forming the major constituent and 9,12-octadecadienoic acid, ethyl ester ($C_{20}H_{36}O_2$, 23.6%), respectively.

In fig. 1, the peak at a retention time of 23.576 min has a mass spectrum data consistent with hexadecanoic acid, ethyl ester. The mass spectrum exhibited a parent ion at *m/z* 284 consistent with the molecular formula $C_8H_{36}O_2$. In addition, in fig. 2, the peak at a retention time of 30.438 min has a mass spectrum data consistent with 9,12-octadecadienoic acid, ethyl ester. The mass spectrum exhibited a parent ion at *m/z* 308 consistent with the molecular formula $C_{20}H_{36}O_2$.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Phellinus linteus* to inhibit 100% of the bacterial growth

Bacterial strains	Concentration (mg/ml)	
	MIC	MBC
S. epidermidis ATCC12228	0.5±0.00	1.0±0.00
P. acnes DMST14916	0.5±0.00	1.0±0.00

MIC: the minimum inhibitory concentration; MBC: the minimum bactericidal concentration (mean±SD)

Table 3: Characterized chemical constituents of *P. linteus* extracts

Compound	RT	PT	MW	MF
Hexadecanoic acid, Ethyl ester	23.57	74.90	284.27	$C_8H_{36}O_2$
9,12-Octadecadienoic acid, Ethyl ester	30.483	23.60	308.27	$C_{20}H_{36}O_2$

RT: retention time (min); PT: percentage (%); MW: molecular weight; MF: molecular formula

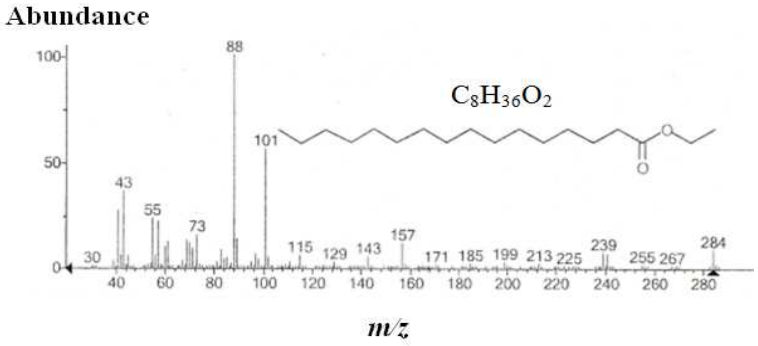

Fig. 1: Mass spectrum of hexadecanoic acid, ethyl ester

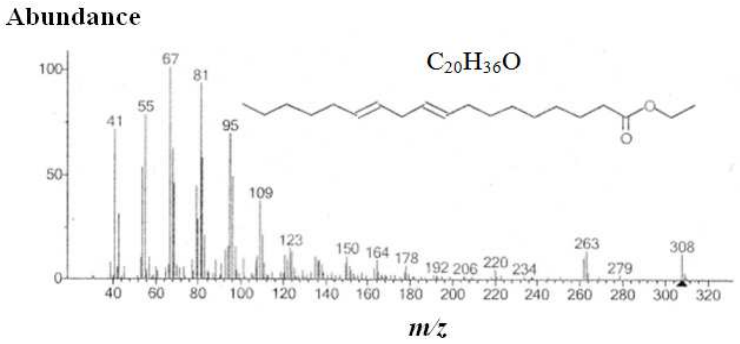

Fig. 2: Mass spectrum of 9,12-octadecadienoic acid, ethyl ester

Previously, many species of Phellinus were reported as producing bioactive compounds like polysaccharides [22], flavones, triterpenes, aromatic acid, and amino acids [14]. These results were similar to those reported by Chinese scientists discovering active compounds from Phellinus species including palmitoleic acid, linoleic acid, oleic acid, hexadecanoic acid, and stearic acid [23].

CONCLUSION

According to the study, the results revealed that the ethanolic extract of *Phellinus linteus* fruiting bodiy possessed measurable antimicrobial activities against both *S. epidermidis* ATCC12228 and *P. acnes* DMST14916. The results also showed MIC and MBC at concentrations of 0.5 mg/ml and 1.0 mg/ml, respectively. However, in order to use these mushrooms properly in medicine, more detailed studies need to be conducted.

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CONFLICT OF INTERESTS

Declared none

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