Original Article

Antibiofilm activity and bioactive phenolic compounds of ethanol extract from the *Hericium erinaceus* basidiome

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Abstract

Biofilm formation has become a serious health and environmental problem. Mushrooms are now considered a valuable source of bioactive compounds with antimicrobial properties. The lion's mane mushroom (*Hericium erinaceus* [HE]) has been used as an antimicrobial for ulcers and gastritis in East Asian countries. However, studies on the antibiofilm activities of HE basidiome against biofilm-forming pathogenic bacteria and their bioactive compound profiles are still limited. The purpose of this study was to determine the antibiofilm activity of HE and to identify its phenolic compound profile. The HE inhibitory activities against bacterial growth and biofilm formation were performed against Pseudomonas aeruginosa, *Salmonella* Typhimurium, Proteus mirabilis, and Staphylococcus aureus. Remarkably, *P. mirabilis* was the most susceptible bacteria to HE. The total phenolic content (TPC) of HE was 1652 ± 1.06 μg/ml, with protocatechuic acid and p-coumaric acid being the most abundant phenolic compounds as determined by high-performance liquid chromatography-mass spectrophotometry (HPLC-MS). This research highlights the possibility of HE as an antibiofilm agent that can be developed as a nutraceutical and natural food preservative.

Key words: Biofilm, *Hericium erinaceus*, pathogenic bacteria, phenolic contents

INTRODUCTION

Biofilms are bacterial communities that are attached to surfaces and covered in an extracellular matrix. With the higher resistance of biofilms to antibiotics compared to planktonic forms, the treatment of infections and food contamination associated with biofilm-forming bacteria has become more challenging.[1,2]

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Current research has attempted to identify effective natural compounds for the prevention and control of biofilms. Bioactive phenolic acids have been reported to exhibit antibiofilm activities.[3] Hence, mushrooms have become a topic of interest in drug discovery as potential sources of phenolic compounds with antibiofilm activities. Several solvents have been used to extract natural antibiofilm substances.[4] The methanol extract (ME) of *Mycena rosea* inhibited *Pseudomonas aeruginosa* biofilms by up to 50%.[5] Meanwhile, the ethanol extract (EE) of *Marasmius oreades* had higher phenolic levels and biofilm inhibition against *S. epidermidis* and *P. aeruginosa* by up to 90%.[6] The ethyl acetate extract (EAE) of *Hericium* sp. WBSP8 showed antibiofilm activity against *Candida albicans*.[7]

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Previous studies have reported HE as a potential antibacterial agent, including its application to relieve gastric ulcers.[8,9] However, research on the antibiofilm activity of EE from HE basidiome against pathogenic biofilm-forming bacteria is still limited. It has been previously noted that ethanol as an extraction solvent displayed better antibiofilm activity. Therefore, the aims of the work were to determine the antibiofilm activity and phenolic compounds of the ethanol extract of HE basidiome, which may increase its potency as a nutraceutical or food preservative.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Salmonella* Typhimurium, and *Staphylococcus aureus*, were maintained in nutrient agar at 4°C for subsequent experiments.

Mushroom samples and extraction

Basidiome samples were collected from Marayat Farm, Pathumthani, Thailand, from February–March 2021 and identified at the Department of Microbiology, Kasetsart University, Thailand, as HE (Voucher No. HE-01). Basidiome samples (200 g) were cleaned and air-dried before extraction. The samples were soaked in 800 mL of 96% ethanol (1:4, w/v) for 7 days at 25°C in the dark. The extract was concentrated at 40°C in a rotary evaporator, then freeze-dried, and stored at −20°C.[10]

Disk diffusion assay

Aliquots of 100 µl bacterial suspension (10^8 CFU/ml) were spread onto sterile Mueller–Hinton Agar (MHA) plates. After that, 6 mm filter paper disks were impregnated with 10 µl HE (200 mg/ml) or ampicillin as a positive control in 5% DMSO. The disks were placed on MHA surfaces and incubated at 37°C for 24 h, followed by a zone of inhibition (ZOI) measurement.[11]

Biofilm quantification assay

A biofilm quantification analysis was performed using the crystal violet assay (CVA). About 200 µl of bacterial culture in Mueller–Hinton broth (10^8 CFU/ml) was inoculated into 96-well microplates and incubated at 37°C for 42 h. The suspensions were then discarded and rinsed with 250 µl NaCl followed by ethanol. The microplates were then dried and subsequently added to 100 µl of 0.1% crystal violet for 15 min of incubation. The stain was then excluded and rinsed with distilled water before being treated with 200 µl of 30% glacial acetic acid. The absorbance was taken at 550 nm using a microplate spectrophotometer.[6] The biofilm-forming bacteria were distinguished using the cutoff OD (ODc). The ODc is three standard deviations higher than the average of the negative control OD at 550 nm. The classifications were as follows: no biofilm producers (OD ≤ ODc), weak (O. Dc < O. D. ≤2 × ODc), moderate (2 ODc < OD ≤ (4 × ODc), and strong (4 × ODc < OD).[12]

Antibiofilm assay

In brief, a 195 µl bacterial suspension (10^8 CFU/ml) and 5 µl HE were added into 96-well microplates at 3.375–100 mg/ml following incubation at 37°C for 42 h. The antibiofilm activities were measured using the CVA assay by comparing the absorbance of treatments with the negative control.[7]

Total phenolic content

The total phenolic content (TPC) was calculated using Folin–Ciocalteu as described by Nowacka *et al*.[13]

Phenolic compound analysis using high-performance liquid chromatography-mass spectrophotometry

The HE (0.1 g/mL) was dissolved in ethanol: water (20:80) and filtered with a 0.22 µm LC disk. Aliquots of 10 µL were injected into an HPLC Agilent 1200 series with a C-18 column operated at 30°C using mobile phase: (A) 0.1% formic acid in H_2O and (B) 0.1% formic acid in acetonitrile with a flow rate of 0.2 ml/min at 280 nm. The MS analysis was conducted using an Agilent 6420 according to the method by Li *et al*. Identification was accomplished through comparison with commercial standards and library databases.[14]

Statistical test

The experiments were repeated three times and the findings were given as mean ± standard deviation. The statistical analysis was carried out in SPSS version 22 (IBM, USA), with a significance value of $P < 0.05$, using one-way ANOVA followed by Duncan's multiple range test.

RESULTS AND DISCUSSION

Antibacterial activity of *Hericium erinaceus*

The antibacterial activities of HE against biofilm-forming bacteria are shown in Table 1. The highest ZOI was found against *S. aureus* (11.7 mm), followed by *P. mirabilis* (6 mm). It revealed that HE did not show sufficient antibacterial activity to inhibit the growth or kill the tested pathogens compared to the positive control.

Biofilm quantification assay

The results showed that *P. aeruginosa* and *S*. Typhimurium were considered strong biofilm producers, while *P. mirabilis* and *S. aureus* were moderate biofilm producers [Table 2].

The Gram-negative bacteria in this study were mostly classified as strong producers. Pili and natural conjugative plasmids attached to surfaces might improve the biofilm formation of Gram-negative bacteria.[15] However, both Gram bacteria may form biofilms of similar properties.[16]

Antibiofilm evaluation of *Hericium erinaceus*

The antibiofilm activities of HE are presented in Figure 1. In general, HE exhibited antibiofilm activities against all the tested bacteria. Therefore, although HE did not show significant antibacterial activity, it revealed antibiofilm

potential. This result was similar to a study of *M. oreades* EE that exhibited low antibacterial effects but high antibiofilm activity against *E. coli, P. aeruginosa, S. aureus*, and *S. epidermidis*.[6]

The minimum biofilm inhibitory concentration ($MBIC_{50}$) of HE against *P. aeruginosa* was 6.25 mg/ml, while concentrations of >12.5 mg/ml were not significantly different. It might be related to *P. aeruginosa* resistance to HE at higher concentrations. *P. aeruginosa* is a Gram-negative, strong biofilm producer associated with bacteremia pneumonia and urinary tract infections (UTIs) due to its high antibiotic resistance.[6,17] The $MBIC_{50}$ of HE against *S.* Typhimurium was 25 mg/ml, with significant differences at higher concentrations. *S.* Typhimurium is a food-borne biofilm-forming pathogen and is the causative agent of enteric fever infection.[18,19]

The moderate biofilm producer, *P. mirabilis*, is a prevalent source of respiratory, gastrointestinal, and UTIs.[20] *S. aureus* is a Gram-positive bacteria responsible for nosocomial and chronic wound infections.[21,22] These moderate biofilm producers were shown to be more vulnerable to the antibiofilm compounds of HE with an $MBIC_{50}$ of 12.5 mg/ml and a significant difference ($P < 0.05$) between concentration treatments.

Table 3 shows the antibiofilm evaluation of HE using the CVA at higher concentrations than $MBIC_{50}$ (100 mg/ml). The darker color of the wells revealed that the bacteria were strong biofilm producers. A lighter color after the treatments showed that the antibiofilm was more effective. The biofilm inhibition of HE at 100 mg/ml against *P. aeruginosa* and *S. typhimurium* was 68.81% and 68.88%, respectively, whereas for *P. mirabilis* and *S. aureus*, it was 78.18% and 70.77%, respectively. Therefore, *P. mirabilis* is the most susceptible strain.

Bioactive phenolic compounds of *Hericium erinaceus*

The phenolic compounds in the HE with a potential antibiofilm effect were identified using high-performance liquid chromatography-mass spectrophotometry (HPLC-MS), as shown by the peak chromatograms, mass spectra, and identified compounds in Figures 2 and 3 and Table 4.

The HE contained a high TPC (1652 ± 1.06 µg/ml) [Table 4], which was higher than in the HE ME from Portugal (288.25 ± 2.48 µg/100 g).[23] The HE also demonstrated different phenolic compound profiles compared to previous reports. Protocatechuic acid (352.94 µg/ml) and p-coumaric acid (42.05 µg/ml) were the major phenolic compounds of HE, while 2-hydroxybenzoic acid methyl ester and ferulic acid were found as traces. Catechin and succinic acid were also found in the extract. Previous studies found that p-coumaric acid (138.02 µg/100 g), gallic acid (76.25 µg/100 g),

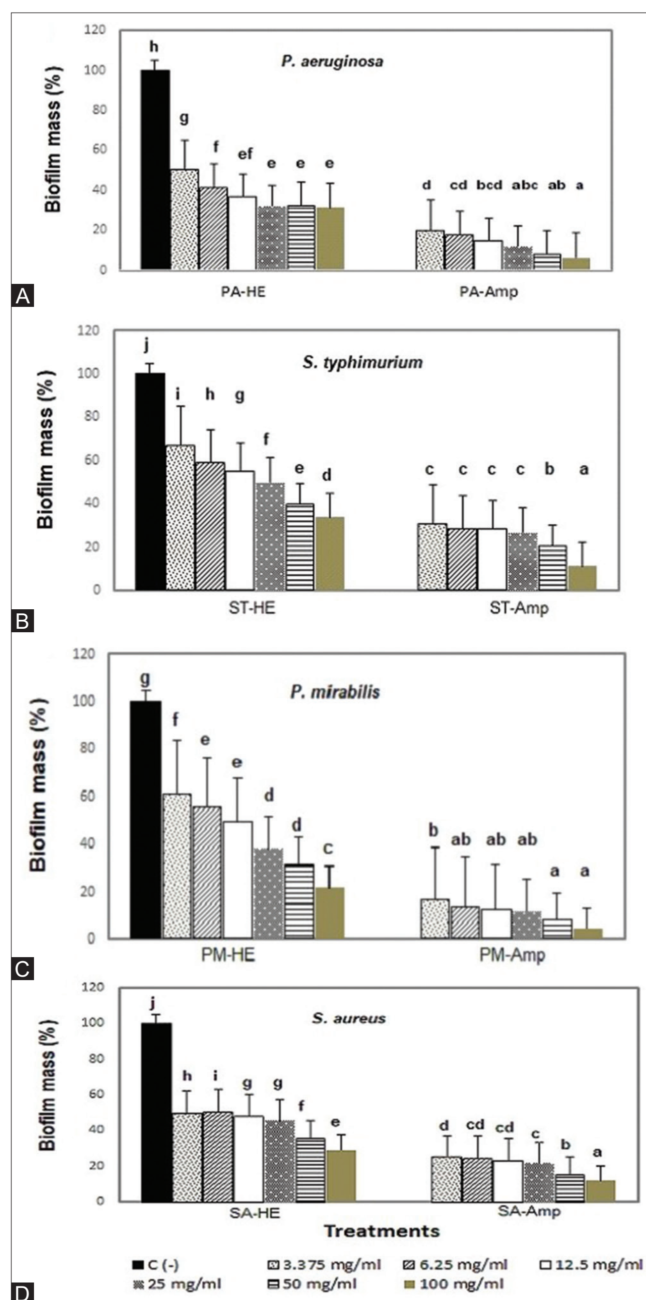

Figure 1: Biofilm inhibition of *Hericium erinaceus* against (A) PA: *Pseudomonas aeruginosa*, (B) ST: *S.* Typhimurium, (C) PM: *Proteus mirabilis*, (D) SA: *Staphylococcus aureus*, Amp: Ampicillin. Means notated by different letters differ significantly ($P < 0.05$)

Table 1: Zone of inhibition of *Hericium erinaceus* (mm±standard deviation)

Treatments	*Pseudomonas aeruginosa*	*S. Typhimurium*	*Proteus mirabilis*	*Staphylococcus aureus*
HE	0_{a}	0^{a}	6 ± 0^{a}	11.7 ± 0.25^{a}
C (+) (ampicillin)	$23.3\pm1.25_{b}$	17.3 ± 0^{b}	23.3 ± 0.5^{b}	38.3 ± 0.25^{b}

Means notated by different letters differ significantly ($P<0.05$). HE: *Hericium erinaceus*

and p-hydroxybenzoic acid (73.99 µg/100 g) were major components in HE ME.[22] HE chloroform extract from Korea comprised ferulic acid (245.83 µg/g), 4-hydroxybenzoic acid (10.88 µg/g), and 4-coumaric acid (2.88 µg/g).[14] These variations could be attributed to the origin of the mushroom strains, cultivation conditions, and solvent used for extraction.

An earlier report demonstrated that the protocatechuic acid and p-hydroxybenzoic acid of *Inonotus obliquus* EE contributed to antibiofilm against *P. aeruginosa* by affecting the bacterial flagella and pili surface attachment, particularly their swimming and twitching ability.[24] Furthermore, ferulic acid and catechin of *M. oreades* EE disrupted *P. aeruginosa* and MRSA biofilms by inhibiting the bacterial motility and physicochemical changes on the surfaces.[6,25] Catechin eradicated the preformed biofilm by decreasing the biomolecule production in the exopolysaccharide biofilms.[26] Succinic acid in *Lentinus edodes* has been reported as a weak antibiofilm against oral bacteria.[27]

Table 2: Classifications of biofilm-forming bacteria used in this study

Bacterial strains	Gram-staining	Classification
Pseudomonas aeruginosa	Gram-negative	Strong
S. Typhimurium	Gram-negative	Strong
Proteus mirabilis	Gram-negative	Moderate
Staphylococcus aureus	Gram-positive	Moderate

There have been few studies on the antibiofilm activity of edible or medicinal mushrooms. This research adds significant information about the antibiofilm of mushrooms that might be useful for health, environmental, and industrial applications.

CONCLUSION

The ethanol extract of HE basidiome had potential antibiofilm activities against pathogenic bacteria, with *P. mirabilis* being the most susceptible. In the HE basidiome,

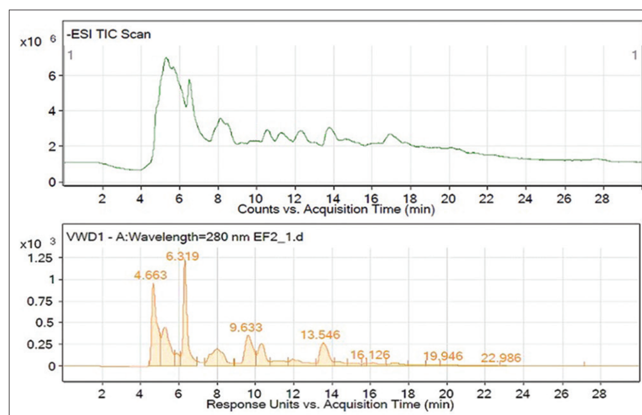

Figure 2: Chromatogram of high-performance liquid chromatography-mass spectrophotometry of *Hericium erinaceus*

Table 3: Antibiofilm evaluation of *Hericium erinaceus* using a crystal violet assay (100 mg/ml)

Gram staining	Bacteria	C (−)	HE	C (+) (ampicillin)
Gram-negative	*Pseudomonas aeruginosa*			
Gram-negative	*S. Typhimurium*			
Gram-negative	*Proteus mirabilis*			
Gram-positive	*Staphylococcus aureus*			

HE: *Hericium erinaceus*

Table 4: Bioactive compounds of *Hericium erinaceus*

RT (min)	Identified compounds	m/z, $(M-H)^-$	Molecular formula	Contents (μg/ml)
4.663	Protocatechuic acid	153	$C_7H_6O_4$	352.94±2.37
6.319	p-coumaric acid	163	$C_9H_8O_3$	42.05±0.05
9.633	Succinic acid	118	$C_4H_6O_4$	ND
13.546	Catechin derivatives	289	$C_{15}H_{14}O_6$	7.96±0.20
16.126	2-hydroxybenzoic acid methyl ester	151	$C_8H_8O_3$	Trace
19.946	Ferulic acid	193	$C_{10}H_{10}O_4$	Trace
	Total phenolic			1652±1.06

ND: Not determined, RT: Retention time

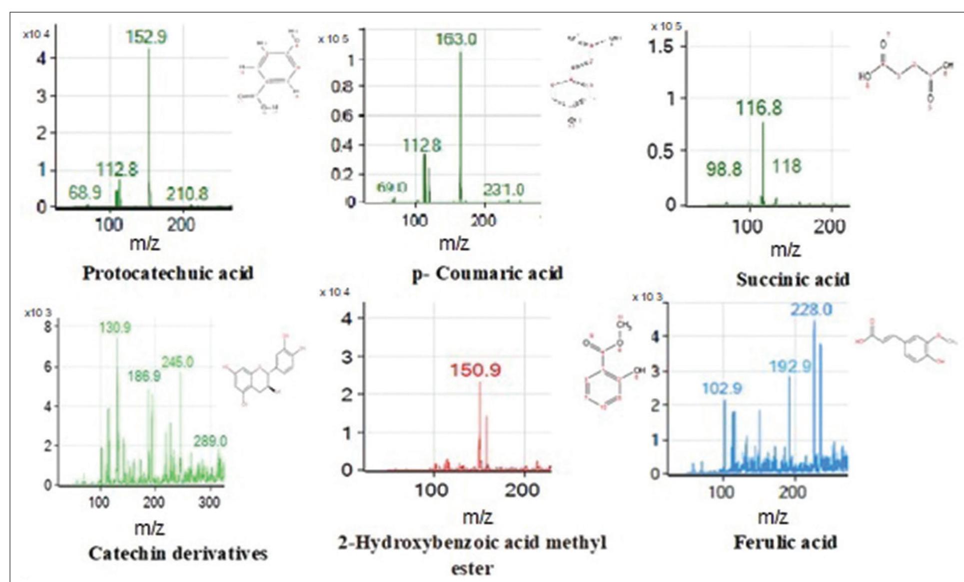

Figure 3: Mass spectra (m/z) of phenolic compounds of *Hericium erinaceus*

proteocatechuic acid and p-coumaric acid were the major phenolic compounds.

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Conflicts of interest

There are no conflicts of interest.

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