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Effects of Cultivation Techniques and Processing on Antimicrobial and Antioxidant Activities of *Hericium erinaceus* (Bull.:Fr.) Pers. Extracts

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Summary

Hericium erinaceus, a temperate mushroom, is currently cultivated in Malaysia. As cultivation and processing conditions may affect the medicinal properties, antimicrobial and antioxidant properties of locally grown *H. erinaceus* have been investigated. The fruitbodies that were fresh, oven-dried or freeze-dried were extracted with methanol. Their properties were compared to those exhibited by mycelium extract of the same mushroom. Various extracts of *H. erinaceus* inhibited the growth of pathogenic bacteria but not of the tested fungus. Mycelium extract contained the highest total phenolic content and the highest ferric reducing antioxidant power (FRAP). The fresh fruitbody extract showed the most potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. However, oven-dried fruitbody extract was excellent in reducing the extent of β-carotene bleaching. The total phenolic content and total antioxidant activity in the oven-dried fruitbody extract was high compared to the freeze-dried or fresh fruitbody extract. This may be due to generation and accumulation of Maillard's reaction products (MRPs), which are known to have antioxidant properties. Thus, the consumption of *H. erinaceus* fruitbody grown in tropical conditions may have health promoting benefits. Furthermore, the production of *H. erinaceus* mycelium in submerged cultures may result in standardized antioxidant formulation for either human nutrition or therapy. Hence, it has been shown that the processing of fruitbody and not the cultivation conditions affects the selected bioactive properties of *H. erinaceus*.

Key words: Hericium erinaceus, antioxidant activity, antimicrobial activity, fruitbody, mycelium

Introduction

Medicinal properties of *Hericium erinaceus* (Bull.: Fr) Pers. (also known as lion's mane, monkey's head, hedgehog fungus, pom pom blanc, and yamabushitake) have been well-known for hundreds of years in traditional Chinese and Japanese cooking and herbal medicine to treat various human diseases. The fruitbody is composed of numerous constituents such as polysaccharides, proteins, lectins, phenols, hericenones, erinacines

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and terpenoids. Some of the biological activities of these components have also been studied (*1*).

A study carried out at the Third People's Hospital of Shanghai showed that *H. erinaceus*, in tablet form, was effective in treating ulcer, inflammation and tumor of the alimentary canal (*2*). The most promising activity of *H. erinaceus* is the stimulation of nerve growth factor (NGF) synthesis by hericenones from the fruitbody and erinacines from mycelium (*3*). It has been shown that aqueous extracts of the mushroom grown in tropical environment could stimulate neurite outgrowth of the cultured cells of the neural hybrid clone NG108-15 (*4*). These findings show that *H. erinaceus* may have a potential in stimulation of neurons to regrow in the treatment of senility, Alzheimer's disease, repairing neurological trauma from strokes, improve muscle/motor response pathways and cognitive function.

In recent years, multiple drug resistance in human pathogenic microorganisms is rampant, due to indiscriminate use of antimicrobial drugs commonly used in the treatment of infectious diseases. In the continuous search for new antimicrobial structures, mushrooms are of interest to investigators (*5–7*). Sixty antimicrobial compounds have been isolated from mushrooms. However, only the compounds from microscopic fungi have been present on the market as antibiotics until now (*7,8*).

Phenolic compounds are one of the most widely distributed plant secondary products. The ability of these compounds to act as antioxidants has been well established. Polyphenols are multifunctional antioxidants that act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers (*9*). Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polyketides, terpenes and steroids. Their phenolic compounds have been found to be excellent antioxidants and synergists that are not mutagenic (*10*). Phenolic antioxidants, such as variegatic acid and diboviquinone, have been found in mushrooms (*11*). Some common edible mushrooms, which are widely consumed in Asian culture, have recently been found to possess antioxidant activity, which was well correlated with their total phenolic content (*11*). Mushrooms may thus be a potential source of natural antioxidants for many applications including the food industry.

Research on the medicinal value of *H. erinaceus* grown in Malaysia, a tropical country, is minimal and yet to be explored. To our knowledge, little or no information is available on the antimicrobial and antioxidant properties of the locally grown mushroom *H. erinaceus*. Cultivation techniques and processing may affect the medicinal properties of *H. erinaceus*. Therefore, the aim of the study is to determine the antimicrobial and antioxidant activities of fresh, freeze-dried and oven-dried fruitbody and mycelium of *H. erinaceus*.

Materials and Methods

Fruiting substrates and preparation of dried fruitbody

In Malaysia, *H. erinaceus* is cultivated on the medium containing rubberwood sawdust, rice bran and calcium carbonate in the mass ratio of 400:8:5. The inoculated bags are placed in a well ventilated mushroom house at (27±2) to (32±2) °C. About 300 g of fresh fruitbody per 800 g of substrate per bag are harvested after 60 days of spawn run (Cheng Poh Guat, personal communication). The fresh fruitbodies were freeze-dried at (–53±2) °C or oven-dried at (50±2) °C for 48 h, then were blended in Waring Commercial Blender (New Hartford, CT, USA) and stored in airtight containers prior to assay.

Culture and mycelium production

A pure culture of *H. erinaceus* obtained from a mushroom farm in Tanjung Sepat, Selangor, Malaysia was grown on potato dextrose agar (PDA, Difco). A volume of 50 mL of dextrose peptone yeast (DPY) liquid medium in 250-mL Erlenmeyer flasks (*12*) was inoculated with 5 mycelium discs from 8-day-old *H. erinaceus* and incubated for 8 days at (26±2) °C and 150 rpm. After incubation, the contents of the flasks were homogenized aseptically in Waring Commercial Blender for 15 s. A volume of 1 mL of 2 % (by volume) mycelium suspension was inoculated into 49 mL of DPY medium and incubated for 10 days at (26±2) °C and 150 rpm. After submerged cultivation, the whole broth was freeze-dried for 48 h at (–50±2) °C.

Preparation of extracts

Biologically active substances from freeze-dried whole broth and fresh, freeze-dried and oven-dried fruitbodies were extracted with 100 % methanol for 24 h at (26±2) °C and 150 rpm. Extracts were filtered through Whatman no. 1 filter paper. The organic solvent in the extracts was removed by a rotary evaporator, and the extracts were kept in the dark at 4 °C for not more than one week prior to analysis for antimicrobial and antioxidant activities.

Antimicrobial activity

The antimicrobial activity of various *H. erinaceus* extracts was tested against Gram-positive bacteria *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *S. aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 7080; Gram-negative bacteria *Salmonella* sp. ATCC 13076, *S. typhimurium*, *Shigella* sp., *S. flexneri* ATCC 12022, *Pseudomonas aeruginosa*, *Escherichia coli* ATCC 29552, *E. coli* strain O157 and *Plesiomonas shigelloides*; and fungi *Candida albicans*, *C. parapsilosis* and *Schizosaccharomyces pombe*.

Antimicrobial activity was studied by using well diffusion method (*13*). Fruitbody and mycelium extracts were dissolved in distilled water to the concentration of 200 mg/mL. Two-day-old cultures of bacteria and fungi were cultured on nutrient agar (Oxoid) and glucose yeast peptone agar plates, respectively. Each plate was divided into four sections and one well in each section was loaded with 40 μL (equivalent to 8 mg) of extract. Distilled water was used as negative control. Chloramphenicol (30 μg/disc) and nystatin (4 μg/well) were used as positive controls for all tested bacteria and fungi, respectively. Three replicate plates were prepared for each species of tested bacteria and fungi. The plates were incubated for 10 h at (37±2) °C, except for *S. pombe*, which was incubated at (27±2) °C, and were then examined for

the presence of clear and hazy inhibition zones. The diameters of the inhibition zones were measured and recorded after 10 h.

Determination of total phenolic content

The concentration of phenolic compounds in the methanol extracts of fruitbody and mycelium, expressed as gallic acid equivalents (GAEs), was determined according to the method described by Cheung *et al.* (*11*). All samples were assayed in triplicate. Butylated hydroxyanisole (BHA) was used as positive control. The gallic acid calibration plot was obtained by plotting the absorbance against the concentration of gallic acid ranging from 25 to 1000 mg/L.

Ferric reducing antioxidant power (FRAP) assay

The modified FRAP assay was performed on 96-well microplates (*14,15*). The working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L of acetate buffer, pH=3.6, with a volume of 10 mmol/L of TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L of hydrocloride acid and with a volume of 20 mmol/L of ferric chloride. A volume of 0.025 mL of methanol extract of each concentration of 4 to 20 mg/mL was added to the wells of 96-well microtiter plate. For each concentration, the test was set up in quadruplicate. Then, 0.175 mL of freshly prepared FRAP reagent at 37 °C were added to three of the replicates, while the same volume of acetate buffer was added to the fourth replicate (blank sample). The plate was placed in an automated microplate reader operated at 593 nm and the temperature was maintained at 37 °C for 4 min. Absorbance values (A_{sample}) were measured after 4 min. Reagent blank reading, using 0.175 mL of FRAP reagent ($A_{reagent\ blank}$), and blank sample reading, using sample and acetate buffer ($A_{blank\ sample}$), were taken. The change in absorbance [A_{sample}– ($A_{reagent\ blank}$+$A_{blank\ sample}$)] was calculated. BHA was used as positive control. The $FeSO_4{\cdot}7H_2O$ calibration plot was obtained by plotting the change in absorbance against 25- to 1000-μM concentrations of $FeSO_4{\cdot}7H_2O$.

Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

The scavenging activity of fruitbody and mycelium extracts on DPPH radical was measured according to the method of Cheung *et al.* (*11*). All samples were assayed in triplicate. BHA was used as positive control. The scavenging activity (%) on DPPH radical was calculated by the following equation:

$$\text{Scavenging activity}=\left(1-\frac{A_{sample}}{A_{control}}\right)\times 100 \qquad /1/$$

The scavenging ability of fruitbody and mycelium extracts was expressed as EC_{50} value (mg/mL), which is the effective concentration at which 50 % of DPPH radicals were scavenged. Low EC_{50} value indicates strong ability of the extract to act as DPPH scavenger.

β-carotene bleaching method

The antioxidant activity of fruitbody and mycelium extracts was determined according to the β-carotene bleaching method described by Cheung *et al.* (*11*). All samples were assayed in triplicate. BHA was used as positive control. The rate of β-carotene bleaching (R) was calculated according to the equation below:

$$R=\frac{\ln(A_0 / A_t)}{t} \qquad /2/$$

where ln is natural logarithm, A_0 is absorbance at time 0, A_t is absorbance at time t, and t is 20, 40, 60, 80, 100 or 120 min. The antioxidant activity was calculated in terms of percentage inhibition relative to the control, using the equation below:

$$\text{Antioxidant activity}=\left(\frac{R_{control}-R_{sample}}{R_{control}}\right)\times 100 \qquad /3/$$

Statistical analysis

The antioxidant data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between the means was determined by the Duncan's multiple range tests at 95 % least significant difference ($p<0.05$).

Results and Discussion

Antimicrobial activity of H. erinaceus extracts

The antimicrobial activity of *H. erinaceus* extracts and standard antibiotics was quantitatively assessed by the presence or absence of clear zones indicating strong inhibition, and hazy (partial) inhibition zones, as given in Table 1.

The fresh fruitbody extract inhibited the growth of tested bacteria, and clear inhibition zones of 8 to 12 mm in diameter against *B. cereus*, *B. subtilis*, *E. faecalis*, *Salmonella* sp., *Shigella* sp. and *P. shigelloides* were observed. No activity was recorded against all the tested fungi. Hazy zones of inhibition with diameters from 13–22 mm, however, were observed against all tested bacteria and fungi (Table 1).

The freeze-dried fruitbody extract also exhibited clear inhibition zones of 8 to 12 mm in diameter against *B. cereus*, *S. aureus* ATCC 6538, *Salmonella* sp., *S. typhimurium*, *Shigella* sp., *P. aeruginosa* and *P. shigelloides*. Furthermore, average hazy zones of inhibition of 13 to 27 mm in diameter against all tested bacteria by the freeze--dried fruitbody extract was observed. The tested fungi were inhibited (hazy zones of 8 to 12 mm in diameter), too.

The oven-dried fruitbody extract exhibited clear inhibition zones of 8 to 12 mm in diameter only against *B. cereus*. Furthermore, hazy inhibition zones were obtained against all tested bacteria and fungi except for *C. albicans*.

Mycelium extract produced clear inhibition zones of 8 to 12 mm in diameter against *B. cereus*, *B. subtilis*, *E. faecalis*, *Salmonella* sp., *Shigella* sp. and *P. shigelloides*. Hazy inhibition zone was, however, demonstrated against all tested bacteria and fungi.

The extracts inhibited four out of five Gram-positive bacteria and five out of eight Gram-negative bacteria tested. In general, Gram-negative bacteria had a higher resistance towards antimicrobial agents and this was ev-

Table 1. Antimicrobial activity of *H. erinaceus* extracts and standard antibiotics

Bacteria/Fungi	Fresh fruitbody	Freeze-dried fruitbody	Oven-dried fruitbody	Mycelium	Chloramphenicol or nystatin
Bacillus cereus	+/3+*	+/3+*	+/3+*	+/4+*	4+[c]
Bacillus subtilis	+/3+*	4+*	4+*	+/5+*	3+[c]
Staphylococcus aureus	3+*	3+*	3+*	5+*	4+[c]
Staphylococcus aureus ATCC 6538	3+*	+/3+*	4+*	3+*	4+[c]
Enterococcus faecalis ATCC 7080	+/3+*	3+*	3+*	+/3+*	3+[c]
Salmonella sp. ATCC 13076	+/3+*	+/3+*	3+*	+/3+*	5+[c]
Salmonella typhimurium	3+*	+/3+*	3+*	4+*	4+[c]
Shigella sp.	+/3+*	+/3+*	3+*	+/5+*	3+[c]
Shigella flexneri ATCC 12022	3+*	3+*	3+*	5+*	5+[c]
Pseudomonas aeruginosa	3+*	+/2+*	3+*	4+*	4+[c]
Escherichia coli ATCC 29552	3+*	3+*	3+*	3+*	4+[c]
Escherichia coli strain O157	3+*	3+*	3+*	3+*	4+[c]
Plesiomonas shigelloides	+/3+*	+/4+*	4+*	+/5+*	5+[c]
Candida albicans	2+*	+*	-	+*	3+[n]
Candida parapsilosis	3+*	+*	+*	+*	3+[n]
Schizosaccharomyces pombe	2+*	+*	+*	+*	2+[n]

Symbols:
* hazy zone; [c] chloramphenicol; [n] nystatin; diameter of inhibition zone: + low level of activity (8 to 12 mm), 2+ 13 to 17 mm, 3+ 18 to 22 mm, 4+ 23 to 27 mm, 5+ high level of activity (28 to 32 mm), – no inhibition

ident from the susceptibility results of *S. flexneri*, *E. coli* and *E. coli* strain O157.

This observation may be explained by the differences in the cell wall structure between Gram-positive and Gram-negative bacteria, as the Gram-negative bacteria possess an outer membrane and a periplasmic space, both of which are absent from Gram-positive bacteria (*16*). The outer membrane is known to present a barrier to the penetration of numerous environmental substances, including antibiotic molecules to the peptidoglycan layer of the cell wall. In addition, the periplasmic space contains enzymes which are capable of breaking down foreign molecules introduced from the outside (*17*). Therefore, the cell walls of Gram-negative bacteria, which are more complex than of the Gram-positive bacteria, act as a diffusional barrier making them less susceptible to the antimicrobial agents than Gram-positive bacteria (*18*).

According to Okamoto *et al.* (*19*), compounds in *H. erinaceus* possess antibacterial and antifungal activities against selected pathogenic microorganisms. Two novel and one known chlorinated orcinol derivatives were isolated from *H. erinaceus* mycelium. These three compounds exhibited antimicrobial activity against *B. subtilis*, *Saccharomyces cerevisiae*, *Verticillium dahliae* and *Aspergillus niger.*

Thin layer chromatography (TLC) revealed that methanol extracts of fruitbody and mycelium of *H. erinaceus* contained various acidic phenol-like and neutral fatty acid-like compounds such as hericenones and hericerins, respectively (*8*). Hericenones were effective against pathogenic microorganisms, while hericerins showed antibacterial activity at low concentrations against *S. aureus*, *B. subtilis* and *E. coli*. To our knowledge, this is the first report of antimicrobial activity of the extracts of variously processed fruitbody and mycelium of *H. erinaceus* against enteric bacteria, including emerging pathogens such as *P. shigelloides* and *E. faecalis*.

Antibiotics could be harmful to beneficial microorganisms in human gut when taken continuously. In this study, the tested microorganisms were partially inhibited by the extracts of *H. erinaceus* as indicated by the hazy zones. Hence, when fresh fruitbody or mycelium extracts are consumed as food or nutraceuticals, the normal flora in the stomach such as *Bifidobacterium adolescentis*, *B. longum* and *Lactobacillus acidophilus*, which protect the intestines from the invasion by pathogens and harmful bacteria, may not be affected (*20*). The extracts however, due to their weak antimicrobial activity, have no potential to be developed into commercial drugs for therapy.

Total phenolic content of H. erinaceus extracts

The total phenolic content, expressed as mg of GAE per gram of fruitbody or mycelium is shown in Table 2. The absorbance value of the tested extract and BHA after subtraction of negative control (y) was translated into total phenolic content using the gallic acid calibration plot with the following formula:

$$\text{Total phenolic content} = \frac{y - 0.00009}{0.0011} \qquad /4/$$

The total phenolic compounds of mycelium were the highest ($p<0.05$) at 31.20 mg of GAE per g of mycelium, followed by oven-dried, freeze-dried and fresh fruitbody. Total phenolic content of BHA was (417.84±28.97) mg of GAE per g of BHA.

Table 2. Total phenolic content and ferric reducing antioxidant power of *H. erinaceus* methanol extracts per g of fruitbody or mycelium

	Total phenolic content	FRAP value
	mg of GAE/g	μmol of $FeSO_4 \cdot 7H_2O$ equivalents/g
Oven-dried fruitbody	$(2.37\pm0.24)^{a}$	$(13.72\pm1.98)^{e}$
Freeze-dried fruitbody	$(0.78\pm0.11)^{ab}$	$(4.06\pm0.30)^{f}$
Fresh fruitbody	$(0.26\pm0.02)^{b}$	$(1.27\pm0.07)^{g}$
Mycelium	$(31.20\pm2.66)^{c}$	$(21.93\pm1.66)^{h}$

GAE, gallic acid equivalents; FRAP, ferric reducing antioxidant power

Values expressed are means±SD of three measurements. Means with different letters in the same column are significantly different ($p<0.05$, ANOVA)

Besides classical antioxidants including vitamin C, E and β-carotene, phenolic compounds have been identified as important antioxidants in mushrooms. Phenolic compounds, or polyphenols, constitute one of the most numerous and widely distributed groups of substances in the plant kingdom. They can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. Flavonoids are reported to be the most abundant polyphenols in human diets (*21*).

Phenolics are one of the major groups of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer (*22*). The reasons for recent renewed interest in phenolics is that most phenolics possess strong antioxidant activity when compared to vitamins C and E *in vitro*, and are readily bioavailable *in vivo* as demonstrated by animal and human studies (*23*). Phenolic compounds in plants are powerful free radical scavengers which can inhibit lipid peroxidation by neutralizing peroxyl radicals generated during the oxidation of lipids (*24*). Since mushrooms also possess phenolic compounds (*11*), it is important to consider the effect of the total phenolic content on the antioxidant activity of mushroom extracts.

Ferric reducing antioxidant power (FRAP) of H. erinaceus extracts

The absorbance value of the tested extract and BHA after subtraction of reagent blank and blank sample was translated into FRAP value (μM of $FeSO_4 \cdot 7H_2O$ equivalents) using the $FeSO_4 \cdot 7H_2O$ calibration plot with the following formula:

$$\text{FRAP value} = \frac{y + 0.0754}{0.0013} \qquad /5/$$

There was a significant difference ($p<0.05$) between the FRAP values of the different extracts (Table 2). Mycelium extract displayed the highest total antioxidant activity of 21.93 μmol of $FeSO_4 \cdot 7H_2O$ equivalents per g of freeze-dried mycelium. The hierarchy of ferric reducing activity per gram of fruitbody was oven-dried fruitbody> >freeze-dried fruitbody>fresh fruitbody (Table 2). FRAP value of BHA was (22618.50±3637.76) μmol of $FeSO_4 \cdot 7H_2O$ equivalents per g of BHA.

Furthermore, there was a weak positive correlation between the antioxidant activity determined by FRAP assay and total phenolic content of the extracts of fruitbody and mycelium (R^2=0.4084), indicating the possible role of the phenolic compounds in the antioxidant activity of *H. erinaceus* (Fig. 1).

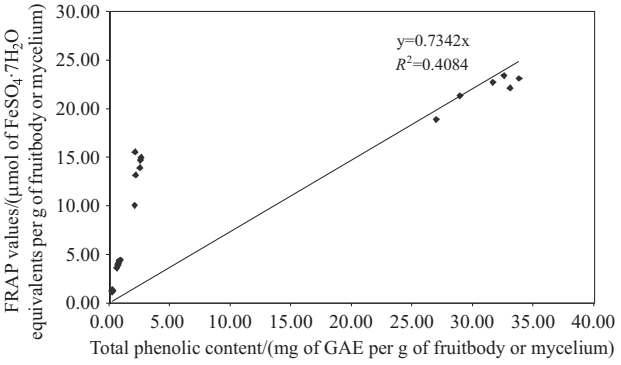

Fig. 1. Correlation between the antioxidant activity determined by ferric reducing antioxidant power (FRAP) assay and total phenolic content of *H. erinaceus* extracts

Various assays are available to evaluate antioxidant potential in food and food-based products. In this study, evaluation of the total antioxidant activity of the extracts of fruitbody and mycelium of *H. erinaceus* was carried out by FRAP assay. It provided a reliable method to study the antioxidant activity of various compounds. This method has been frequently used for a rapid evaluation of the total antioxidant activity of various food and beverages (*25*), different plant extracts containing flavonoids (*26*) and dietary polyphenols and a limited number of flavonoids *in vitro* (*27*). The reaction is nonspecific, and any half-reaction that has a less-positive redox potential, under reaction conditions, than the Fe^{3+}/Fe^{2+}-TPTZ half-reaction will drive Fe^{3+}-TPTZ reduction (*28*). FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue coloured Fe^{2+}--tripyridyltriazine compound from the colourless oxidised Fe^{3+} form by the action of electron donating antioxidants.

Scavenging activity of H. erinaceus extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

H. erinaceus extracts contain active substances, including phenolic compounds that have hydrogen-donating activity to scavenge DPPH radical as a possible mechanism for their antioxidant activity (Fig. 2). Scavenging effects of the fresh, oven-dried and freeze-dried fruitbody on DPPH radical increased up to 87.35, 87.78 and 89.24 % with the increasing concentrations of extracts at 7, 10 and 14 mg/mL, respectively, after which they reached a steady state (Fig. 2). The scavenging effect of mycelium extract also increased with increasing concentrations of the extract, but did not reach steady state at 15 mg/mL of the extract. This implied that the DPPH radical scavenging of the fruitbody and mycelium extracts was dose-dependent. In this study, at 6.4

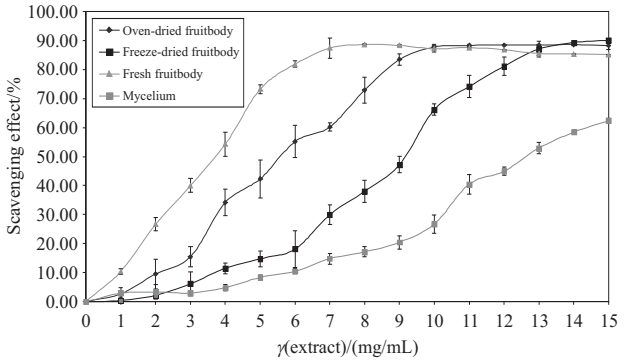

Fig. 2. Scavenging effect of *H. erinaceus* extracts on 1,1,-diphenyl-2-picrylhydrazyl (DPPH) radical. Values are expressed as means±SD of three measurements

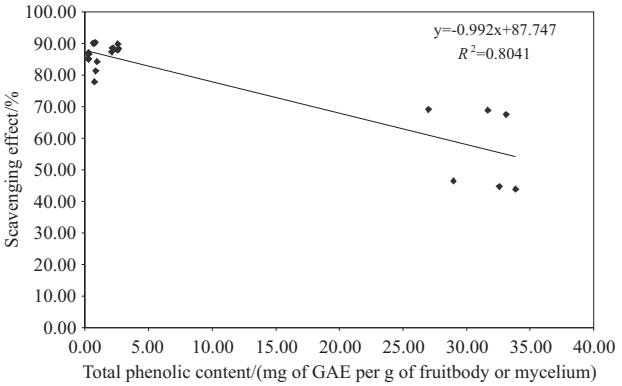

Fig. 3. Correlation between the antioxidant activity determined by scavenging effect on DPPH radical and total phenolic content in *H. erinaceus* extracts

mg/mL of the extract, scavenging effect of the oven-dried fruitbody extract was 56.84 %, compared to 63.2–67.8 % of oven-dried *H. erinaceus*, *G. frondosa* and *T. giganteum* obtained by Mau *et al.* (*29*).

EC_{50} values of the extracts in DPPH radical scavenging of the fresh fruitbody, oven-dried fruitbody, freeze-dried fruitbody and mycelium extracts were approx. 3.75, 5.81, 8.67 and 13.67 mg/mL, respectively, whereas that of BHA was 0.0126 mg/mL (Table 3). Freeze-dried fruitbody and mycelium extracts were not effective in scavenging DPPH radical. Among the tested extracts, that of fresh fruitbody was the best DPPH scavenger with the lowest EC_{50} value, followed by the extracts of oven-dried fruitbody, freeze-dried fruitbody and mycelium.

Table 3. Scavenging ability (EC_{50} values) of *H. erinaceus* extracts and BHA on DPPH radical

	EC_{50} values/(mg/mL)
Oven-dried fruitbody	5.81
Freeze-dried fruitbody	8.67
Fresh fruitbody	3.75
Mycelium	13.67
BHA	0.01261

EC_{50}, 50 % effective concentration

There was a strong negative correlation between the scavenging ability on DPPH radical and total phenolic content in the extracts of fruitbody and mycelium (R^2= 0.5629), as shown in Fig. 3, which indicates that high scavenging ability on DPPH radical is not due to phenolic compounds in *H. erinaceus* extracts.

As compared to commercial and specialty mushrooms, *Auricularia* sp. are good $DPPH^{\cdot}$ scavengers. Methanol extracts of *Auricularia mesenterica* and *Auricularia polytricha* showed an outstanding scavenging effect of 100 % at 1.0 mg/mL of the extract, whereas *Auricularia fuscosuccinea* and *Tremella fuciformis* showed scavenging effects of 95.4 % at 3.0 mg/mL of the extract, and 71.5 % at 5.0 mg/mL of the extract, respectively (*30*). *Auricularia fuscosuccinea* scavenged DPPH radical by 94.5 % at 0.4 mg/mL of the extract (*31*). Excellent scavenging effects (96.3 to 99.1 % and 97.1 %) were also observed with methanol extracts of *Antrodia camphorata* and *Agaricus brasiliensis* at 2.5 mg/mL of the extract, respectively. Scavenging effect of methanol extracts of other medicinal mushrooms was measured at up to 0.64 mg/mL and it was 24.6, 67.6, 74.4 and 73.5 % for *Coriolus versicolor*, *Ganoderma lucidum*, antler-shaped *Ganoderma lucidum* and *Ganoderma tsugae*, respectively (*31*).

The scavenging activity of mushroom extracts was tested using a methanol solution of the 'stable' free DPPH radical. Unlike laboratory-generated free radicals such as hydroxyl radical and superoxide anion, DPPH radical has the advantage of being unaffected by side reactions, such as metal ion chelation and enzyme inhibition (*32*). Extracts of *H. erinaceus* were free radical inhibitors or scavengers, acting possibly as primary antioxidants. The extracts might react with free radicals, particularly peroxy radicals, which are the major propagators of the autoxidation chain of fat, thereby terminating the chain reaction (*29*).

β-carotene bleaching activity of H. erinaceus extracts

Table 4 shows the antioxidant activity of the extracts and BHA with the coupled oxidation of β-carotene and linoleic acid. The antioxidant activity of BHA gradually increased with the increasing concentration of BHA ($p<0.05$). The antioxidant activity of the extracts, however, varied with different concentration of the extract depending on how the fruitbody was processed. Oven-dried and freeze-dried fruitbody extracts exhibited higher antioxidant activities than the extracts of fresh fruitbody and mycelium. At the concentration of 20 mg/mL of the extract, the antioxidant activity was in the following order: oven-dried fruitbody>freeze-dried fruitbody>fresh fruitbody>mycelium.

It is probable that the antioxidative components in the mushroom extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. The mechanism of β-carotene bleaching is a free-radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene, in this model system, undergoes rapid discolouration in the absence of an antioxidant. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic

Table 4. Antioxidant activity of *H. erinaceus* extracts and BHA measured by β-carotene bleaching method

γ(extract) mg/mL	Antioxidant activity				
	Oven-dried fruitbody	Freeze-dried fruitbody	Fresh fruitbody	Mycelium	BHA
4	$(43.21\pm2.92)^{ap}$	$(36.67\pm1.16)^{aq}$	$(15.35\pm1.46)^{ar}$	$(14.64\pm3.76)^{ar}$	$(58.34\pm1.14)^{as}$
8	$(47.40\pm2.89)^{bp}$	$(49.26\pm1.44)^{bp}$	$(30.00\pm3.05)^{bq}$	$(25.44\pm2.76)^{br}$	$(78.27\pm0.75)^{bs}$
12	$(62.93\pm2.33)^{cp}$	$(53.10\pm2.35)^{cq}$	$(45.92\pm1.25)^{cr}$	$(25.91\pm2.45)^{bs}$	$(85.22\pm0.77)^{ct}$
16	$(59.22\pm2.39)^{dp}$	$(54.87\pm1.51)^{cp}$	$(27.65\pm2.83)^{bq}$	$(30.66\pm3.81)^{cq}$	$(89.30\pm0.46)^{dr}$
20	$(60.67\pm1.18)^{cdp}$	$(60.04\pm1.87)^{dp}$	$(38.17\pm2.13)^{gq}$	$(29.45\pm1.32)^{bcr}$	$(99.13\pm0.38)^{es}$

Values expressed are means±SD of three measurements. For the same extract with different concentrations, means in the same column with different letters (a–e) were significantly different ($p<0.05$, ANOVA). For different extracts with the same concentration, means in the same row with different letters (p–t) were significantly different ($p<0.05$, ANOVA)

methylene groups, attacks the highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically (*33*). The presence of antioxidant extracts can hinder the extent of β-carotene bleaching, neutralising the linoleate-free radical and other free radicals formed within the system (*34*). Hence, this forms the basis by which mushroom extracts can be screened for their antioxidant potential.

Effect of cultivation and processing conditions on the selected bioactive properties of H. erinaceus

Traditionally, *Hericium erinaceus* prefers cool climate such as in the northern part of Thailand, central part of Taiwan and southeastern coast of China (*35*) to grow and produce fruitbody. However, this mushroom can be grown in tropical climates, too. Cultivation of *H. erinaceus* in tropical conditions (high temperatures and humidity) in Malaysia did not affect the production of selected bioactive properties as shown in this study and in a previous one (*4*). Mycelium biomass produced under standard conditions in submerged fermentation, which requires sterile handling technology and more technical demands, is now becoming increasingly important especially for nutraceutical and pharmaceutical production (*36*). Furthermore, environmental conditions can be optimized and controlled to ensure the quality and quantity of active ingredients of the mycelium biomass, and subsequently the extract.

Promising results were obtained with the methanol extracts of fresh and freeze-dried fruitbody and mycelium, which to varying extents inhibited the growth of the tested microorganisms. Fungi, however, were not sensitive to any of the tested extracts. Extracts of oven-dried fruitbody lost their antimicrobial activity, which could be due to inactivation *via* oxidation of the bioactive compounds that were responsible for antimicrobial activity at (50±2) °C. Therefore, heat-processed mushrooms may have lower or no antimicrobial activity compared to the corresponding fresh and freeze-dried forms. Freeze dried fruitbody was able to maintain not only its form after the water had been evaporated, leaving it dry and easy to store for long periods of time, but caused minimal damage to the bioactive compounds (*37*). However, for commercial purposes, this could be an expensive processing method.

It is well known that natural nutrients could be significantly lost during the thermal processing due to the fact that most of the bioactive compounds are relatively unstable to heat. Temperature during drying and heating process affects compound stability due to chemical and enzymatic decomposition, or losses by volatization or thermal decomposition. These latter have been suggested to be the main mechanism causing the reduction of polyphenol content (*38*). In some cases, however, heat treatment caused no change or even improved the content and activities of naturally occurring antioxidants. Moreover, novel compounds having antioxidant property, such as Maillard's reaction products (MRPs), may be formed as a result of heat treatment. Therefore, the loss of natural antioxidants or heat labile nutrients can be minimized by an enhancement of overall antioxidant activity in plant food due to their various chemical changes during heat treatment (*38*). The most likely explanation for the higher total antioxidant activity in oven-dried fruitbody is an increased antioxidative principle due to the generation and accumulation of antioxidant compounds and MRPs during the heating process.

It has been reported that a prolonged heating time (30 min) and higher heating temperature (121 °C) significantly enhanced the overall antioxidant activity of *Lentinula edodes* (*38*). This could be explained by the increased amount of antioxidant compounds, especially free polyphenolic compounds. Heat-treated *Lentinula edodes* (*38*) and oven-dried fruitbody of *H. erinaceus* mushroom may have increased the beneficial effects on health associated with the increase of antioxidant activity. However, only two mushroom varieties have been studied so far. Further studies with different mushrooms are needed to validate matrix effects. The formation of phenolic compounds during the heating process might be due to the availability of precursors of phenolic molecules by non-enzymatic interconversion between phenolic molecules subjected to the effects of external factors, such as temperature. Thus, the mushroom composition and the degree of heating could be important factors contributing to high total polyphenol content. However, further investigation is needed to truly explain this phenomenon. Increased heat treatment led to development of antioxidant activity, which has positive effects on hu-

man health, but browning that occurred during heating was not desirable to consumers. Hence, a balance between positive and negative effects should be taken into account before simulating their formation during processing (*39*).

Conclusions

It has been concluded from the results of this investigation that the extracts of *H. erinaceus* are weak inhibitors of bacterial and fungal growth. However, bacteria were found to be more sensitive to the bioactive compounds compared to fungi. Furthermore, the inhibition was dependent on the type of the tested extracts and the oven-dried fruitbody extract did not have antimicrobial activity.

The use of different methods in antioxidant activity assessment is necessary. The present study showed that no single testing method was sufficient to estimate the antioxidant activity of a sample. The combination of three methods applied in this study gave valuable information to evaluate the antioxidant activity of *H. erinaceus* and could be recommended for other similar investigations. The most widely used methods for measuring antioxidant activity are those that involve the generation of radical species, where the presence of antioxidants caused the reduction or removal of radicals.

Heat treatment of fruitbody liberated phenolic compounds and increased generation of Maillard's reaction products (MRPs), and thus increased the amounts of active compounds present in the extract. Oven drying of mushrooms for storage at predetermined temperature may be a process to concentrate the antioxidant activity of fresh mushrooms. Therefore, oven-dried fruitbody could be used as food or incorporated as food ingredient in determined amounts to replace artificial antioxidants as possible protective agents in human diets to reduce oxidative damage.

In this study, it was shown that the processing of fruitbody and not the cultivation conditions affected the selected bioactive properties of *H. erinaceus*. Full investigation on the protocol of drying is also necessary for this mushroom when cultivated on a large scale. However, whether such extracts will act as effective therapeutic agents remains to be investigated. Prior to the application of these extracts by the nutraceutical/pharmaceutical industry, the identification of the active compounds and the study of mechanisms of actions are highly recommended.

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