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Effect of Crude Extract from Mycelium and Fruiting Body of *Isaria tenuipes* BCC 31640 on Tyrosinase Inhibition and Antioxidant Activities

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Abstract

This work investigated the effect of crude extract from mycelium and fruiting body of *Isaria tenuipes* BCC31640 on tyrosinase inhibition and antioxidant activities. *I. tenuipes* BCC31640 was cultivated in liquid and solid mediums at different times. The researchers determined the mycelium wet weight and dry weight at 60°C for 24 h. These samples were extracted using 80% methanol. The highest tyrosinase inhibition exhibited in fruiting body was cultivated in solid media for 42 days (IC_{50} ~ 0.0426 ± 0.0224 mg/ml) compared with Kojic acid (IC_{50} ~ 0.0642 ± 0.0399 mg/ml). The results revealed that the solid and liquid media and cultivation time showed significant effects on antioxidant activities and tyrosinase inhibition. The antioxidant activities were determined by the DPPH method. This study showed that the effect of crude extract from mycelium of *I. tenuipes* BBC 31640 cultivated in liquid media for 7 days gave the highest of antioxidant activities (IC_{50} ~ 0.6195 ± 0.0097 mg/ml) compared with L-ascorbic acid (IC_{50} ~ 0.0581 ± 0.0114 mg/ml).

Keywords: *Isaria tenuipes*, Antioxidant Activities, Tyrosinase Inhibition, Mycelium, Fruiting Body

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1. Introduction

At present, many bioactive compounds that were isolated from fungi are used as medicines and pesticides. Bioactive molecules produced from fungi (mushroom) mainly belong to polysaccharide, glucans, terpenoids, phenolic compounds, lectins, statins, etc. [1]. They have immune-modulating, antioxidant, genoprotective, antitumor, hypocholesterolemic, antidaibetic, hepatoprotective and other medicinal effects [2-3]. High antioxidant activities provide health benefits in preventing damages due to free radicals produced by biological degeneration [4]. Recently, antioxidants have attracted considerable attention in relation to radicals and oxidative stress, cancer prophylaxis and therapy, and longevity [5]. Phenols and polyphenols are the target analytes in many such cases; they may be detected by enzymes like tyrosinase or other phenol oxidases, or even by plant tissues containing these enzymes [6-8]. Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase enzyme involved in melanogenesis [9]. The enzyme is widely distributed in fungi, higher plants and animals, and is involved in the first two steps of the melanin biosynthesis, in which L-tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine (L-DOPA, monophenolase activity) and the latter is subsequently oxidated to dopaquinone (diphenolase activity). Tyrosinase inhibitors such as arbutin, kojic acid and hydroquinones have been used as whitening or antihyperpigment agents because of their ability to suppress dermal-melanin production [10]. However, arbutin and kojic acid hardly showed inhibitory activity against pigmentation in intact melanocytes or in a clinical trial, and hydroquinones are considered to be cytotoxic to melanocytes and potentially mutagenic to mammalian cells [11]. Therefore, it remains necessary to search for new tyrosinase inhibitors without side effects.

Polysaccharides protect neuronal cells against the free radical-induced cellular toxicity and stimulate steroidogenesis. Polysaccharides possess significant immune-stipulating, antitumor, antioxidant, antibacterial and antiviral activities. Fungal terpenoids (tri- and sesquiterpenes) have cycotoxic, antibacterial, antifungal, hypocholesterolemic, hypoglycemic, hypotensive antioxidant effects. Chitin and chitosan isolated from fungal cell walls are regulating the functions of livers, gastro-intestinal tracts and kidneys [12-13]. Fungal pigment melanin possesses antioxidant, immune-modulating, anti-mutagenic and radioprotective properties [2]. Bioactive proteins, lectins and hydrophobins are carbohydrate-binding proteins and most interesting for various medicinal applications, such as increasing biocompatibility of medicinal implants devices, immobilization of antibodies in a biosensor and stabilizing oil vesicles for drug delivery [14].

Genus Isaria belongs to phylum Ascomycota and class Sordariomycetes. Many Ascomycetes species have been used as the source of disease combating natural products with

tremendous biological, pharmacological and immunomodulatory activities [15-17].

Isaria tenuipes (formerly *Paecilomyces tenuipes*, also known as *Isaria japonica*) has long been consumed as traditional health food and folk medicine in Japan, Korea and China as it offers various types of biological and pharmacological activities, such as immunomodulatory [18], anti-tumor [19], antidepressant [20] and hypoglycemic action [21]. Polysaccharides extract from the mycelium of these fungi constitute the main bioactive agents and exhibit multiple pharmacological activities including antitumor, anti-inflammatory, immunopotentiation, hypoglycemic and hypocholesterolemic effects, protection of neuronal cells against the free radical-induced cellular toxicity, steroidogenesis and antioxidant activities [4]. Some valuable components, such as polysaccharides [22] nucleoside, protein [23], cordyceps acid [24], cordycepin, tenuipesine, sterol, trichothecanes [25] and cyclopeptide [26] have been found from *I. tenuipes* [27]. Polysaccharides possess great potential and considered as important tool for studying the development of nutraceutical products. However, under liquid culture conditions, the productivity of bioactive compounds has been observed to vary with environmental conditions (medium composition, carbon source, nitrogen source, pH, etc.). Commercial cultivation through a liquid culture is becoming quite useful nowadays because of higher mycelial yield with fewer chances of contamination [4], [28], [29]. In view of this, present studies have been investigated the effect of crude extract from mycelium and fruiting body of *I. tenuipes* BCC 31640 on tyrosinase inhibition and antioxidant activities.

2. Materials and Experiment

2.1 Source of Inoculum and Culture Media Studies

I. tenuipes BBC 31640 was obtained from the BIOTEC culture collection laboratory, National Center for Genetic Engineering and Biotechnology, Thailand. The fungal was transferred into potato dextrose agar and incubated at 25 °C for 14 days. The fungal suspension was pipetted onto petri dishes containing potato dextrose agar-based solid medium (PDA: 15 g agar, 15 g dextrose, 200 g potato tubers in 1 L of dH_2O) and spread using a Drigalsky spatula. The plates were incubated at 25 °C in a total darkness for 15 days. The inoculum consisted of fungal colony disc of about 1 cm diameter, cut up using cork borer after the pure culture had been grown on a complete medium.

2.2 Liquid (Submerge) Culture Media

Liquid cultivation was done in a modified culture medium (potato broth 200 g/L, rice broth 200 g/L, yeast powder 5.0 g/L, dextrose 15 g/L, molasses 30 g/L, KH_2PO_4 0.36 g/L, $NaHPO_4.7H_2O$ 1.05 g/L, $MgSO_4 \cdot 7H_20$ 0.6 g/L, KCl 0.1 g/L, $NaNO_3$ 1.58 g/L [30]. The 100 ml liquid medium was prepared and placed into each bottle. The bottles were autoclaved at a temperature of 121 °C, pressure 15 lb/in^2 for 15 min. The 2 fungal colonies' disc was about 1 cm diameter was inoculate in each bottle then incubated in total darkness for 21 days. The samples were taken

every 3 days for observed mycelium mass production. The researchers determined the mycelium wet weight and dry weight at 60 °C for 24 h. The wet and dry weight of the mycelium mass was expressed in a g.bolttle^{-1}.

2.3 Solid Culture Media

The solid media was prepared from the standard medium (whole rice) and other modified culture medium as mention above. The whole rice 40 g and modified culture medium of 40 ml were prepared and placed into each bottle. The bottles were autoclaved at a temperature of 121 °C, pressure 15 lb/in^2 for 15 min. 4% of inoculum was transferred (v/w) into each bottle then incubated in total darkness for 35 days. The samples were taken every 7days for observed fruiting body production. The wet and dry weight of the fruiting body was expresses in a g.bolttle^{-1}.

2.4 Sample Preparation

After fermentation, the mycelium and fruiting body were harvested by filtration. The combine mycelium (residue) was washed well with distilled water and then tray dried at 60 °C until constant weight. The dry weights of mycelium and fruiting body were accurately measure. The dried mycelium and fruiting body were pulverized. The tyrosinase inhibition and antioxidant activity of *I. tenuipes* BBC 31640 were crude extracted from the pulverized by warm water at 45 °C with extracting time 12 h and solid-liquid ratio 1:50 (w/v) then 95% (volume fraction) ethanol was added to it with a final concentration of 80%(v/v). After this extraction, the solutions were filtered through Whatman no.1 filter paper. The solvents were evaporated by rotary evaporator at a temperature of 45 °C, 120 rpm, pressure 100 mbar for quantitative analysis.

2.5 Bioactive Compounds Assays

2.5.1 Tyrosinase Inhibition Assays

Tyrosinase inhibition assays were performed with dihydroxyphenylalanine (L-DOPA) as substrate. The reaction mixture (1000 μL) contained 685 μL of phosphate buffer (0.05 M, pH 6.5), 15 μL of mushroom tyrosinase (2500 U mL^{-1}), 200 μL of plant extract solution and 100 μL of 5 mM L-DOPA. After the addition of L-DOPA the reaction was immediately monitored at 492 nm for dopachrome formation in the reaction mixture. Kojic acid is a well-known tyrosinase inhibitor and used as a positive control. The concentration range of extract used for the mushroom tyrosinase inhibition assay was 0–0.3 mg/mL. Each measurement was made in triplicate. The IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of concentration-response curves [6].

2.5.2 Antioxidant Assays

Determination of the antioxidant. The total free radical-scavenging molecules was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) methods [31]. For this , DPPH (200 μm) solution at different concentration (2-10 mg/mL) was added to 0.05 mL of the samples dissolved in ethanol. An equal amount of ethanol was added to the control. Ascorbic acid was used as the control. The absorbance was read after 20 min.at

517 nm and the inhibition was calculated using the formula:

$$I\,(\%) = (A_{blank}\text{-}A_{sample})/\,A_{blank} \times 100,$$

Where A_{blank} was the absorbance of the control reaction and Asample was the absorbance in the presence of the sample.

The activity was expressed as a concentration of necessary samples to give a 50% reduction in the original absorbance (IC_{50}). Each experiment was performed in triplicate.

3. Results and Discussion

3.1 Effect of Liquid and Solid Media and Cultivation Time for *I. tenuipes* BBC 31640 Production

To find the effect of liquid and solid media and cultivation time for *I. tenuipes* BBC 31640 production, *I. tenuipes* BBC 31640 was grown in the liquid and solid culture media at different time. The cultivation times showed significant effects on average mycelium and fruiting body weight (Table 1 and Table 2). The liquid media reached the maximum level of 10.01 g.bolttle^{-1} mycelium production after 21 days of fermentation while the fruiting body reached a maximum levels of 14.23 g.bolttle^{-1} after 35 days. The production time of fruiting body requires longer than that of mycelium to complete all process. The results obtained in this study corresponds to Sharma *et al* [32] that the cultivation through a liquid culture is becoming quite useful nowadays because of higher mycelium yield.

Table 1 Effect of Liquid Media for Mycelium Production

Days	Wet weight (g.bolttle^{-1})	Dried weight (g.bolttle^{-1})	Moisture (%)
3	1.45^{a}	0.06^{a}	95.00
6	2.80^{b}	0.21^{b}	92.50
9	4.34^{c}	0.46^{c}	89.40
12	6.05^{d}	0.83^{d}	87.93
15	7.30^{e}	1.21^{e}	83.42
18	8.32^{f}	1.45^{f}	82.57
21	10.01^{g}	1.66^{g}	83.41

Table 2 Effect of Liquid Media for Fruiting Body Production

Days	Wet weight (g.bolttle^{-1})	Dried weight (g.bolttle^{-1})	Moisture (%)
7	2.10^{a}	0.66^{a}	68.57
14	3.60^{b}	1.01^{b}	71.94
21	6.64^{c}	2.35^{c}	64.60
28	10.43^{d}	3.30^{d}	68.36
35	14.23^{e}	4.26^{e}	70.06

[a]Each measurement is the mean of three replications ± one standard deviation. Means within a column with different letters (a,b,c,d) are significantly different at $P < 0.05$

[a,b,c,d]Dependent variables:lipid; different letters refer to significantly different lipid contents at 95% confidence intervals; identical letters refer to insignificantly different lipid contents at 95% confidence intervals.

3.2 Effect of Culture Media for Antioxidant Activities

Antioxidants are compounds capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. DPPH (2,2-diphenyl-1-

picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH does not dimerize, as happens with most free radicals. The delocalization on the DPPH molecule determines the occurrence of a purple colour, with an absorbtion band with a maximum around 520 nm.

The IC_{50} values of mycelium and fruiting body extracts are shown in Table 3. The antioxidant activities of the fruiting body were higher than mycelium. The IC_{50} values of mycelium at 7 days gave the highest antioxidant activity because of the values nearest to the standard and the fruiting body gave the highest antioxidant activity at 28 days compared with the standard (IC_{50} ~ 0.058 mg/ml). The longer cultivation time decreases the antioxidant activity of both the mycelium and the fruiting body. The results revealed that the culture media showed significant effects on antioxidant activities.

Table 3 Antioxidant Activities of Mycelial and Fruiting Body Extracts

	Days	DPPH assay [IC_{50} (mg/ml)]
Mycelium	7	0.6195 ± 0.0097^{b}
Mycelium	14	0.8199 ± 0.2116^{c}
Mycelium	21	0.7863 ± 0.1670^{c}
Fruiting body	28	1.4193 ± 0.1870^{b}
Fruiting body	35	2.3066 ± 0.9730^{c}
Fruiting body	42	3.7999 ± 0.5190^{d}
L-ascorbic acid	Control	0.0581 ± 0.0114^{a}

[a]Each measurement is the mean of three replications ± one standard deviation. Means within a column with different letters (a,b,c,d) are significantly different at $P < 0.05$

[a,b,c,d] Dependent variables:lipid; different letters refer to significantly different lipid contents at 95% confidence intervals; identical letters refer to insignificantly different lipid contents at 95% confidence intervals.

3.3 Effect of Culture Media for Tyrosinase Inhibition

Table 4 shows the inhibition of tyrosinase activity of mycelium, fruiting body and kojic acid (positive control) at 0.064 mg/ml. The results indicated that the tyrosinase inhibition of mycelium at 7 days was higher than mycelium at 21 days whereas the tyrosinase inhibition of the fruiting body at 42 days was higher than the fruiting body at 28 days. This meant that the short cultivation time for mycelium gave the highest tyrosinase inhibition while the longer cultivation time for the fruiting body gave the highest tyrosinase inhibition

Table 4 Tyrosinase inhibition of crude extracts mycelium and fruiting body

	Days	Tyrosinase inhibition [IC_{50} (mg/ml)]
Mycelium	7	0.1812 ± 0.0649^{b}
Mycelium	14	0.7055 ± 0.0634^{c}
Mycelium	21	0.6472 ± 0.5345^{c}
Fruiting body	28	0.1904 ± 0.0324^{b}
Fruiting body	35	0.0536 ± 0.0375^{a}
Fruiting body	42	0.0426 ± 0.0224^{a}
Kojic acid	Control	0.0642 ± 0.0399^{a}

[a]Each measurement is the mean of three replications ± one standard deviation. Means within a column with different letters (a,b,c,d) are significantly different at $P < 0.05$

[a,b,c,d] Dependent variables:lipid; different letters refer to significantly different lipid contents at 95% confidence intervals; identical letters refer to insignificantly different lipid contents at 95% confidence intervals.

4. Conclusion

The interest in finding the effect of solid and liquid media and cultivation time on antioxidant activities and tyrosinase inhibition produced from *I. tenuipes* BBC 31640 was analyzed. *I. tenuipes* BBC 31640 extracts showed significant antioxidant activities. The mycelium cultivated in liquid media for 7 days gave the highest antioxidant activities (IC_{50} ~ 0.6195 ± 0.0097 mg/ml) compared with L-ascorbic acid (IC_{50} ~ 0.0581 ± 0.0114 mg/ml) and the fruiting body cultivated in solid media for 28 days gave the highest antioxidant activities (IC_{50} ~ 1.4193 ± 0.1870 mg/ml). In addition, the longer cultivation time decreased antioxidant activities both in mycelium and fruiting body. For tyrosinase inhibition, the cultivation time of mycelium in liquid media for 7 days gave the highest tyrosinase inhibition (IC_{50} ~ 0.1812 ± 0.0649mg/ml) compared with Kojic acid (IC_{50} ~ 0.0642 ± 0.0399 mg/ml) whereas the cultivation time of the fruiting body in solid media for 42 days gave the highest tyrosinase inhibition (IC_{50} ~.0.0426 ± 0.0224 mg/ml). This study showed that the effect of crude extract from mycelium of *I. tenuipes* BBC 31640 cultivated in liquid media for 7 days gave the highest of both antioxidant activities and tyrosinase inhibition. The inhibition of tyrosinase has an important role in order to prevent melanin accumulation in skin. Therefore, tyrosinase inhibitors are an attractive target in cosmetics and treatments for pigmentation disorders [6].

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