

Bioactive compounds and antioxidant activity of Lion's Mane mushroom (*Hericium erinaceus*) from different growth periods

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Abstract. Lion's Mane mushroom (*Hericium erinaceus*) is one of the most popularly edible and medicinal mushrooms. However, there is still a lack of knowledge on the relationship between growth period and bioactive content in the mushroom. The objectives of this research were to study bioactive compounds and antioxidant activity of Lion's Mane mushroom at various growth periods. The mushroom was cultivated and harvested at growth periods of 14, 21, and 28-days. The samples were dried by lyophilization and extracted with ethanol. Bioactive compounds (ergosterol, hericenone C, and hericene A), total phenolic content, total flavonoid content, and antioxidant activity of the samples were analyzed. HPLC analysis demonstrated the highest concentration of ergosterol, hericenone C and hericene A in the 14-days, 21-days, and 28-days samples, respectively. Total phenolic content and total flavonoid content of the dried sample were not statistically significant different ($p > 0.05$). The 21-days sample showed higher activity than the 14-days and 28-days samples for both DPPH and ABTS radical scavenging assays. In conclusion, the 14-days sample showed the highest concentrations of bioactive compounds, while the 21-days sample showed the highest yield and antioxidant activity. The 28-days sample exhibited a change in morphology and color. This study demonstrates that the growth periods of Lion's Mane mushroom play a role in their bioactive compounds and antioxidant activities. Finally, the correlation of growth periods to the content of other bioactive compounds can be used for mushroom cultivation and applications in foods, food supplements and cosmetics.

Keyword. Hericenone C, Ergosterol, Hericene A, ABTS, DPPH

1 Introduction

Mushrooms are good sources of nutrients and more than 2000 species of edible and/or medicinal mushrooms were identified [1]. Mushroom intake receives very high attention due to its flavour, beneficial health properties, and high nutritional value especially protein [2, 3]. Some examples of edible mushrooms are the White button mushroom (*Agaricus bisporus*), Maitake mushroom (*Grifola frondosa*) and Enoki mushroom (*Flammulina velutipes*). Several mushrooms are sources of health-promoting compounds, and some are medicinal mushrooms such as Lion's Mane mushroom (*Hericium erinaceus*), Reishi mushroom (*Ganoderma lucidum*) and Chaga mushroom (*Inonotus obliquus*), which indicate high potentials for many health benefits, such as hyperglycemia effect, improved lipid metabolism, anti-obesity, anti-inflammatory, anti-proliferative, and antioxidant capacity [4-7].

Lion's Mane mushroom (*Hericium erinaceus*), LM, also known as Pompom mushroom, Monkey Head mushroom, Yamabushitake mushroom (Japanese) and Houtou mushroom (Chinese), is one of the medicinal mushrooms that contains many bioactive compounds [8]. This mushroom looks like a pack of white silk thread (5-20 cm) and grows in oak, walnut, and perennials. Generally, Lion's Mane mushroom are found in the Northern Hemisphere, such as Europe and East Asia, especially in Japan [9]. Bioactive compounds from Lion's Mane mushroom have many health benefits, such as neuroprotective effects, anti-obesity, antioxidant, anti-microbial, anti-inflammatory, and improved immunology [10-16]. Antioxidant activity from several compounds, such as phenolic compounds, flavonoid compounds, and unique bioactive compounds has been demonstrated. It can reduce brain degeneration in people with mild cognitive impairment in both mice and the

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elderly and exhibits more stimulating effects on the nervous system than other mushrooms [17, 18].

Mushroom cultivation is an important profession in the Thai economy. In many cases for commercialization, agricultural wastes, e.g. rice straw, are utilized as substrates for mushroom cultivation, which is well agreed to the circular economy. Nowadays, the development of mushroom cultivation is important for control and preferable production. Farm environments and growth substrates significantly affect the quantity and quality of the mushroom obtained [19-22]. There are many studies on the effects of humidity, light, temperature, and growth substrate on bioactive compounds and the activity of the mushroom but only a few studies on the effect of growth period were reported. Antioxidant activity, total phenolic content, and total flavonoid content in each growth period of the giant *Leucopax* mushroom (*Leucopaxillus giganteus*) mycelia were demonstrated only at a laboratory scale [23]. In this study, Lion's Mane mushroom were cultivated in a farm with controlled environments, in terms of temperature and humidity, and the bioactive compounds in the mushroom from different growth periods were investigated. The information obtained will help the mushroom farm or processor to choose the optimal growth period of the mushroom for sale or further product development. The control of cultivation conditions would relate to the sustainable development of natural resource management.

2 Material and methods

2.1 Raw materials and chemicals

Lion's Mane mushrooms were cultivated at Fresh & Friendly Farm Co., Ltd., Pathumthani province, Thailand. Ethanol (AR grade) was obtained from PanReac AppliChem ITW Reagents (Barcelona, Spain), while ergosterol was supplied by Sigma-Aldrich (Madrid, Spain). Gallic acid was bought from Sigma-Aldrich (USA). Folin-Ciocalteu reagent, 2,2'-azino-bis (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sisco Research Laboratory Pvt. Ltd. Sodium carbonate (Na₂CO₃), sodium nitrite (NaNO₂), sodium hydroxide (NaOH), aluminium chloride (AlCl₃) and potassium persulfate (K₂S₂O₈) were purchased from Merck (India). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Copens Scientific (M) Sdn Bhd (Tedia, USA), while water (HPLC grade) and methanol (HPLC grade) were supplied from LAB-SCAN (Gliwice, Poland).

2.2 Lion's Mane mushroom cultivation

In this study, Lion's Mane mushroom was cultivated under controlled conditions including temperature and humidity. The room temperature was controlled at 15-16°C throughout the cultivation. The cultivation parameters including carbon dioxide concentration were monitored continuously for 28 days by corresponding sensors in the inoculation room via the online system.

The growth substrates were composed of sawdust, rice bran, and corn cob at the ratio of 40:30:30, and their acidity was adjusted to pH 9-10 using calcium carbonate (CaCO₃). After sterilization, the growth substrate in the plastic bottles was inoculated with Lion's Mane mushroom spawn and inoculated for their mycelium growth for 18-20 days. The growth substrate composts were opened to let the fruiting bodies grow in the inoculation room. Lion's Mane mushrooms were harvested and determined for their morphology, yield, bioactive compounds, and antioxidant activity at 14-days, 21-days, and 28-days of cultivation.

2.3 Preparation of Lion's Mane mushroom

Lion's Mane mushrooms harvested from different growth periods were lyophilized using a freeze drier (GFD30S, Grisrianthong, Thailand) at -30 °C for 36 h. The dried samples were ground and separated into small sizes (particle size < 150 µm). One hundred mL of ethanol was added to 5 g of the dried sample and extracted for 72 h in the dark. The ethanol extracts were filtered by filter papers (Whatman[®] qualitative filter paper, Grade 1) and dried by a rotary vacuum evaporator (Rotavapor[®] R114, Buchi, Switzerland). The crude extract was stored at -4 °C until use. The yield percentage of the dried mushroom powder was calculated as g dried LM/100g fresh LM while % crude extract was calculated from the crude extract (g) obtained from 100 g of dried LM. The moisture content of the dried Lion's Mane mushroom was analyzed by followed AOAC 1999 [24].

2.4 Analysis

2.4.1 Determination of bioactive compounds

The samples were dissolved in methanol for the analysis of total phenolic content, total flavonoid content, bioactive compounds, and antioxidant activity. The crude extracts in methanol were filtered by filter membranes (PES 0.22 µm 13 mm Chromplus) before the analysis by high performance liquid chromatography (HPLC, Agilent 1200, Germany). Column C18 column (250 x 4.6 mm, 5 µm particle size, Zorbax Eclipse Plus C18, Agilent) with a guard column (12.5 x 4.6 mm, 5 µm particle size, Zorbax Eclipse Plus, Agilent) were used. The analysis was conducted at 25 °C, with a UV detector at a wavelength of 282 nm. The mobile phase was methanol: water of 98:2. Ergosterol standard was used as a reference for quantification. The content of ergosterol, hericenone C, and hericene A were calculated from their peak area compared with the area of ergosterol standard.

2.4.2 Total phenolic content

Each extract was measured for its total phenolic content by Folin-Ciocalteu (FC) method followed by Cheung, Cheung, & Ooi 2003 [25] with slight modification. Ten

μL of crude extract and 8 μL of Folin-Ciocalteu reagent were loaded onto a 96-well microplate. After 5 min, 20 μL of 20% sodium carbonate was added and made up to 200 μL with methanol. The reaction was measured for absorbance at 750 nm by a microplate reader (iMark™ Microplate Absorbance Reader, BIORAD, Japan). The results were calculated as gallic acid equivalent (GAE)/g dried LM.

2.4.3 Total flavonoid content

Total flavonoid content in the samples was determined by aluminium chloride assay followed by Moon *et al.* 2018 [26] with slight modification. Ten μL of crude extract and 10 μL of 5% sodium nitrite were loaded onto 96-well microplates containing 170 μL of distilled water. After 6 min, 10 μL of aluminium chloride and 50 μL of 1 M sodium hydroxide were added, after 5 min incubation. The absorbance at 540 nm was measured using a microplate reader (iMark™ Microplate Absorbance Reader, BIO RAD, Japan). The results were calculated as catechin equivalent (CE)/g dried LM.

2.4.4 Antioxidant activity

Antioxidant activity of the samples was analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and 2,2'-azino-bis (ABTS) radical scavenging assay followed by Gasecka *et al.* 2020 [13] with slight modification. DPPH radical scavenging activity of the samples was determined by adding 100 μL DPPH reagent and 50 μL of the extracts onto a 96-well microplate. After 30 min, the reaction was measured for absorbance at 540 nm by a microplate reader (iMark™ Microplate Absorbance Reader, BIO RAD, Japan). For ABTS radical scavenging assay, 7mM ABTS was prepared in 5 mL distilled water and 175 μL of 140 mM potassium persulfate was added to the flask and made up to 10 mL with distilled water. After 16 h, 50 μL of crude extract and 100 μL of ABTS solution were loaded onto a 96-well microplate. After 30 min, the reaction was measured for absorbance at 750 nm by a microplate reader (iMark™ Microplate Absorbance Reader, BIORAD, Japan). The DPPH and ABTS radical scavenging activity were calculated as follows (1):

$$\% \text{ Inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100 \quad (1)$$

where A_{control} is the absorbance of the control that contained all reagents except the samples. A_{sample} is the absorbance of the samples with reagents added.

The radical scavenging activity from DPPH and ABTS radical scavenging assay were expressed as IC₅₀ value (mg extract/mL), which is the inhibitory concentration at which 50% of radicals were scavenged. A low IC₅₀ value indicates the strong activity of the sample.

2.5 Statistical analysis

The results were expressed as the mean value of three

replicates (n=3) and standard deviation. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test.

3 Results and discussions

3.1 Cultivation and preparation of dried Lion's Mane mushroom

Environmental conditions during the cultivation of Lion's Mane mushroom were monitored using corresponding sensors and recorded (Table 1). The growth periods in this study were shorter than 35-days in the study of Imtiaj *et al.* 2008 [21], and similar to Hassan 2007 [27], who reported the cultivation for 14–20 days. Different cultivation conditions and growth substrates significantly affect the growth periods and morphology of the mushrooms. According to the standard and mushroom specification of Fresh & Friendly Farm, Lion's Mane mushrooms should present a white exterior, short white silk thread, and firm texture. The optimal morphology of the cultivated mushroom was observed at 14-days, while 21-days and 28-days samples showed more yellow colour, irregular shape, and long yellow silk (Table 2). The yield of the dried Lion's Mane mushroom at 14-days, 21-days, and 28-days cultivation were in a range of 13.5-16.17% (g/100g dried LM) as presented in Table 3. The 28-days sample indicated the lowest yield of dried Lion's Mane mushroom which might be due to the accumulation of water within their cells. However, crude extract content from all growth periods was not significantly different (p>0.05).

Table 1. Environmental conditions during the cultivation of the Lion's Mane mushroom farm.

Cultivation conditions	Cultivation time (day)	
	0-7	8-28
Temperature (°C)	16.17±0.18	15.30±0.61
Humidity (%)	91.37±2.32	88.64±3.33
Light (lx)	4.27±0.16	0.82±0.37
Carbon dioxide (ppm)	538±197	542±116

3.2 Bioactive compounds

HPLC chromatograms of 14-days, 21-days, and 28-days LM extracts show similar peak and retention times of bioactive compounds (Figure 1). Hericenone C, ergosterol, and hericene A were found at 14.9 min, 17 min, and 28.54 min of retention time, respectively. We have previously isolated and purified hericenone C and hericene A from Lion's Mane mushroom and confirmed the compounds by NMR (Nuclear magnetic resonance). Both purified compounds were identified for their retention times by HPLC previously (data not shown). The results showed that the highest content of hericenone C, ergosterol, and hericene A were detected

at 14-days as 1.23 ± 0.10 mg/g dried LM, 3.54 ± 0.30 mg/g dried LM, 0.09 ± 0.01 mg/g dried LM and decreased to 0.73 ± 0.09 mg/g dried LM, 2.62 ± 0.31 mg/g dried LM, 0.03 ± 0.01 mg/g dried LM and 0.48 ± 0.01 mg/g dried LM, 1.87 ± 0.06 mg/g dried LM at 21-days and 28-days, respectively, as presented in Table 4. Hericenone C and Hericene A are bioactive compounds specifically found in Lion's Mane mushroom. Hericene A were not detected at 28-days of cultivation. Bioactive compounds were produced in the early stages of the growth period 14-days and decreased after that. Ergosterol content found in this study, 3.54 mg/g dried LM, was much higher than ergosterol content reported by Gasecka *et al.* 2020 [13], 4.5 mg/g dried LM.

Table 2. Fresh mushroom, dried powder, and suspension in ethanol of Lion's Mane mushroom from different growth periods.










Lion's Mane mushroom	Growth periods		
	14-days	21-days	28-days
Fresh mushroom			
Dried LM powder			
Suspension in ethanol			

Table 3. Yield, moisture, total phenolic contents, and total flavonoid content in Lion's Mane mushroom from different growth periods

Yields and compositions	Growth periods (days)		
	14	21	28
Yield (g dried LM/100g fresh LM)	14.35	16.175	13.5
Crude extract ^{ns} (g dried extract/100g dried LM)	11.73±1.13	14.58±0.58	13.38±1.89
Moisture (% dry weight basic)	9.46±0.29 ^a	7.27±0.08 ^b	6.79±0.09 ^c
Total phenolic content ^{ns} (mg GAE/g dried LM)	0.85±0.15	0.90±0.19	0.87±0.23
Total flavonoid content ^{ns} (mg CE/g dried LM)	3.66±1.90	2.92±0.79	3.19±0.73

*Different superscripts in the same row mean significant difference at $p < 0.05$

^{ns}No significant differences at $p < 0.05$

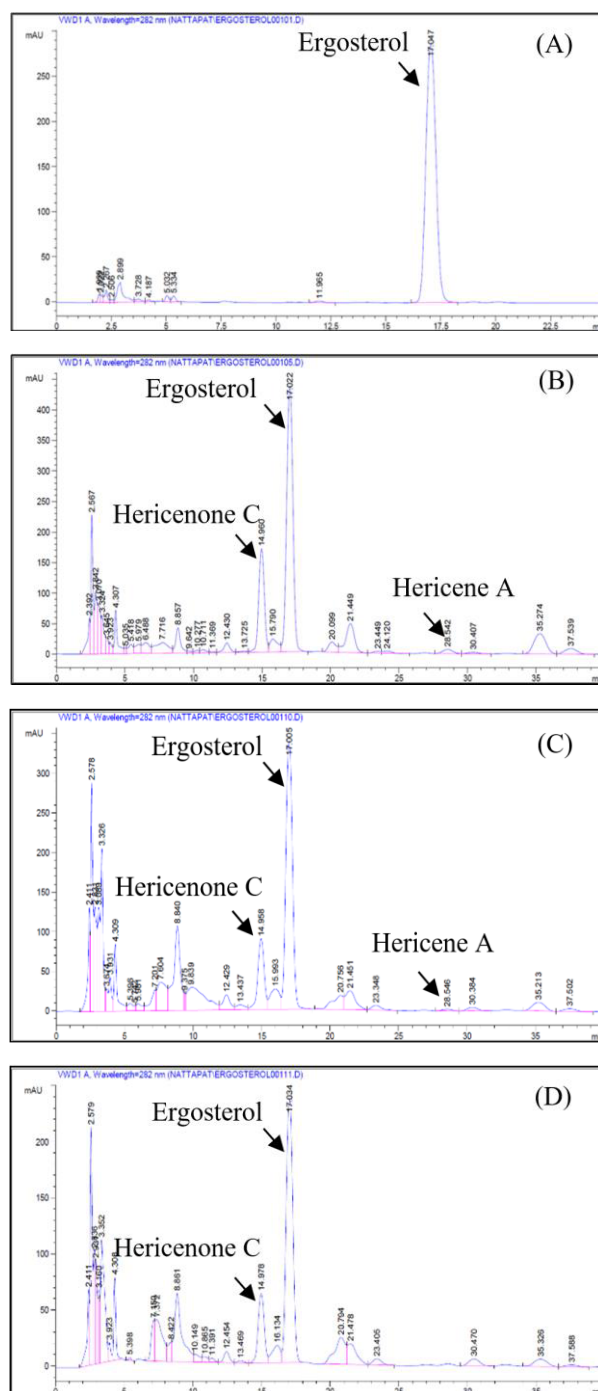


Figure 1. HPLC chromatogram of: ergosterol standard (A); and Lion's Mane mushroom from each growth periods, 14-days (B), 21-days (C) and 28-days (D).

3.3 Total phenolic content and total flavonoid content

Total phenolic content and total flavonoid content in the Lion's Mane mushroom from different growth periods were not significantly different ($p > 0.05$) as presented in Table 3. The content of phenolic and flavonoid compounds tended to decrease along the growth period from 14-days to 28-days. The compounds may change their forms during each growth period [28]. The total phenolic content of the samples in this study was similar

to the data demonstrated by Wong *et al.* 2009 [10]. However, the total phenolic content of the samples in our study was lower than wild Lion's Mane mushrooms [7]. Both total phenolic content and total flavonoid content from giant leucopax mushroom mycelia were reported to increase with increasing growth periods [23]. Different results for the effect of the growth periods on both total phenolic content and total flavonoid content might be due to the different mushroom species and cultivation environments in the mushroom farm.

Table 4. Bioactive compounds in Lion's Mane mushroom from different growth periods.

Bioactive compounds	Growth period*		
	14 days	21 days	28 days
Ergosterol (mg/g dried LM)	3.54±0.30 ^a	2.62±0.31 ^b	1.87±0.06 ^c
Hericenone C (mg/g dried LM)	1.23±0.10 ^a	0.73±0.09 ^b	0.48±0.01 ^c
Hericine A (mg/g dried LM)	0.09±0.01 ^a	0.03±0.01 ^b	-

*Different superscripts in the same row mean significant difference at $p < 0.05$

3.4 Antioxidant activity

The inhibition capacity, IC_{50} measured by DPPH and ABTS radical scavenging activity of the 21-days sample was higher than those of 14-days and 28-days samples (Figure 2). The degree of antioxidant capacity (IC_{50}) of the samples in this study, 63.30 mg extract/mL were lower than 5×10^{-3} mg extract/mL of the Lion's Mane mushroom harvested from nature [7]. The correlation between DPPH and ABTS assays was found with total phenolic content as reported by Dudone *et al.* 2009 [29]. However, the antioxidant capacity in our study did not correlate well with the total flavonoid content in our study. Therefore, it is possible that there are other compounds not identified in this study, which can exhibit antioxidant activity such as β -glucans, caffeic acid, ergosterol derivatives, and hericenone derivatives in the extract. However, what exactly the compounds are should be investigated in further study. The antioxidant activity of Lion's Mane mushroom samples in this study is different from the giant leucopax mushroom mycelia. The growth periods of the giant leucopax mushroom mycelia correlated with the increase of antioxidant activity were demonstrated by Barros *et al.* 2007 [23].

4 Conclusions

Lion's Mane mushrooms were cultivated in the farms under controlled temperature and humidity. The research presented that the bioactive compounds of Lion's Mane mushrooms from different growth periods, including hericenone C, ergosterol and hericine A were highest at

14-days cultivation and decreased to lower content after that (21-days and 28-days). However, antioxidant activity measured by 2 different methods, total phenolic content and total flavonoid content were not significantly different between each growth period of the mushroom. The knowledge obtained from this study could be used for the cultivation as well as the criteria to select the proper harvesting time to achieve the mushroom with standard shape and health benefit compounds for further applications. The results and information obtained from the research would be beneficial to the production of high-quality mushrooms for consumption and to be used for value-added products. The farm management for maximum yield and high-quality products will also enhance sustainable development and natural bioresource management.

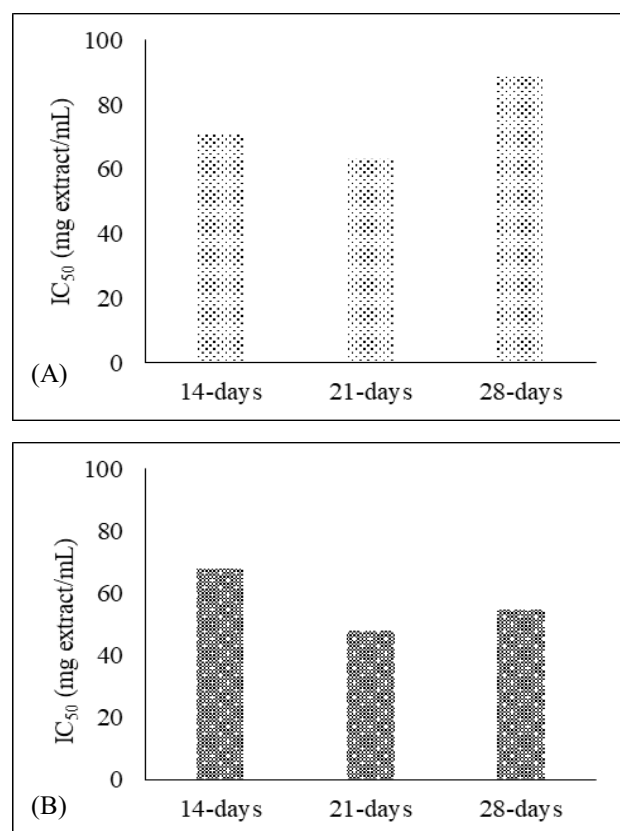


Figure 2. DPPH radical scavenging assay (A), and ABTS radical scavenging assay (B) of Lion's Mane mushroom from different growth periods.

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