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IN VITRO ANTIOXIDANT ACTIVITY AND BIOACTIVE COMPOUNDS FROM *Calocybe indica*

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Abstract. Nowadays, the use of mushrooms in medicine is ubiquitous and has achieved particular success. The antioxidants in mushrooms can deactivate free radicals. This study assesses the antioxidant potential of mushroom *Calocybe indica* with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging methods and the total antioxidant capacity. The mushroom's ethanol extract exhibits acceptable activity with a low IC_{50} value (240.11 μg/mL), approximately 2.9 times lower than that of the mushroom *Ophiocordyceps sobolifera* extract. The ABTS scavenging rate of the extract is around 60% at 500 μg/mL, and the total antioxidant capacity is equivalent to 64.94 ± 1.03 mg of GA/g or 77.42 ± 0.42 μmol of AS/g. The total phenolics, flavonoids, polysaccharides, and triterpenoids are equivalent to 29.33 ± 0.16 mg of GAE/g, 17.84 ± 0.11 mg of QUE/g (5.04 ± 0.04%), and 4.96 ± 0.04 mg of oleanolic acid/g, respectively. Specifically, the total triterpenoid content has been reported for the first time. The mushroom can have potential biomedical applications.

Keywords: *Calocybe indica*, antioxidant activity, polysaccharide, triterpenoid, total phenolics, total flavonoid

1 Introduction

Antioxidant activity is one of the most commonly considered essential biological activities in food or medicinal use for disease prevention and treatment. Oxygen active forms, such as free radicals and oxygen-containing molecules with high oxidizing activity are $OH^{\cdot}$, HOO^{-}, and O^{2-}, have high energy and are unstable. They readily attack macromolecules, such as lipids, DNA, and proteins, destroying cells, causing mutations, cancer, cardiovascular diseases, diabetes, obesity, and accelerated ageing [1, 2]. Therefore, an appropriate quantity of antioxidants in the human body can prevent some accidents, slow down the aging process of the body, protect the integrity of cells, liver function, and the function of the nervous system, limit inflammatory agents and treat Alzheimer's and Parkinson's diseases [3-5].

There exist several experimental methods to determine the antioxidant activity [1, 6, 7]. These methods differ in terms of their assay principle, experimental conditions, and reaction mechanism. The approaches based on the stable free radical from 2,2-diphenyl-1-picrylhydrazyl

(DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and total antioxidant capacity are the most effective for determining antioxidant activity because of their rapidity and simplicity [6].

Mushrooms are a food source with unique flavours and contain essential biologically active compounds with immunomodulatory, antioxidant, and inhibitory properties [8, 9]. The mushroom (*Calocybe indica* – Nam Trang Sua) indigenous to India and belonging to genus *Calocybe* and family Lyophyllaceae was first described by Purkayastha and Chandra [10, 11]. This mushroom is a Vietnamese common kind of food and is considered "clean meat" with relatively high protein content, vitamins, and minerals [10]. It is a valuable kind of food and traditional medicine. The species contains antioxidant [12-14], inhibits cancer cells, and prevents metastasis [15], inflammation caused by UV radiation [16], proliferation, apoptosis, and migration [15]. Recently, researchers have discovered numerous compounds with biological activities and secondary metabolites from *Calocybe indica* and documented their chemical composition [16-18]. However, to the best of our knowledge, there is limited information about *in vitro* antioxidant activity and chemical constituents of *Calocybe indica* from Vietnam. Therefore, in this study, we focus on evaluating the antioxidant capacity of *Calocybe indica* with the ABTS radical scavenging and DPPH radical scavenging methods.

2 Experimental

2.1 Plant material, chemicals and equipment

***Materials*:** *Calocybe indica* was collected in June 2020, in Phu Luong, Phu Vang, Thua Thien Hue, Vietnam, and taxonomically identified (Fig. 1). A voucher specimen is deposited at the Department of Chemistry, University of Sciences, Hue University.

Chemicals and equipment: Ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich Co. (USA). Gallic acid, quercetin, sulfuric acid, ammonium molybdate, and sodium phosphate were supplied from Shandong Chemical Co. (China). The ethanol used in all experiments was food grade and purchased from local suppliers. Other reagents and solvents were of analytical grade. The Jasco V-630 Spectrophotometer (Japan Spectroscopic Company, Japan) was used to determine the absorbance of the samples.

2.2 Ethanol extract

The dried mushroom was pulverized and passed through a 200-mesh sieve. The powder sample (20 g) was immersed in 500 mL of ethanol three times at 78 °C for 3 hours. The solutions were combined, filtered, and evaporated under reduced pressure at 50 °C, yielding a crude ethanol extract (approximately 4.97 % (w/w)). The resulting crude extract was then stored at –20 °C, for further analysis (without polysaccharide) [19].

Fig. 1. Calocybe *indica* grown at the study site

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2.3 Total antioxidant activity with phosphor-molybdenum method

A quantity of aliquot of the sample (0.3 mL) was mixed with 3 mL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Next, the mixture was incubated at 95 °C for 90 min. The mixture was then cooled to 25 °C, and the absorbance was measured at a wavelength of 695 nm against a blank containing 3 mL of the reagent solution without sample [20]. The antioxidant activity was evaluated from the optical density of the sample. The high optical density value indicates that the sample exhibits high antioxidant activity [20]. The antioxidant capacity is equivalent to the quantity of gallic acid [21] or ascorbic acid [22] (the standard curve equation for gallic acid and ascorbic acid is Abs = 1.951 × C_{GA} + 0.2373; R = 0.9994 and Abs = 4.7727 × C_{AS} – 0.0055; R = 0.9999).

2.4 Evaluation of DPPH radical scavenging activity

One and a half millilitres of 100–500-μg/mL extract was dissolved in 1.5 mL of 100 μM DPPH in ethanol. The reaction mixture was shaken for one minute and incubated at ambient temperature for 30 minutes. Then, its optical density (OD) was measured at a 517-nm wavelength. Ethanol was used as a blank sample and ascorbic acid as a positive control (reference standard) [23]. The inhibition of the DPPH radicals in per cent was calculated as follows:

Inhibition of DPPH (%) =
$(OD_{DPPH} – OD_{sample+DPPH})/OD_{DPPH} \times 100$

The radical scavenging activity was evaluated from the IC_{50} value, which is the concentration of an extract causing 50% of DPPH radical inhibition (calculated from the inhibition percentage/concentration equation).

2.5 ABTS radical scavenging assay

The ABTS radical was generated by mixing ABTS (7 mM) with potassium persulfate (2.45 mM) in the dark at ambient temperature for 16 h [24]. A quantity of sample (0.1 mL) with 100–500 μg/mL concentrations was mixed with 3.9 mL of $ABTS^{+\cdot}$ solution, and the absorbance was measured at 734 nm. Ascorbic acid was used as a positive control. The scavenging capability towards $ABTS^{+\cdot}$ was calculated as follows:

Scavenging rate (%) = $(1 - A_1/A_0) \times 100$

where A_0 and A_1 is the absorbance of the blank and the sample. The radical scavenging activity was evaluated from the IC_{50} value.

2.6 Total phenolic content

A volume of the ethanol extract solution (0.5 mL) was mixed with 2.5 mL of the Folin–Ciocalteu (1:10) and 2 mL saturated Na_2CO_3 solution. The tubes were incubated for two hours at ambient temperature for colour development. The solution's optical density was determined at a 760-nm wavelength. The results were expressed as milligrams of gallic acid equivalents (GAE) per one gram of sample [25] (the standard curve equation of gallic acid is Abs = 9.396 × C_{GA} – 0.0652; R = 0.9959).

2.7 Total flavonoid content

One millilitre of the ethanol extract solution was diluted with a mixture of 4 mL of deionized water and 0.3 mL of 5% $NaNO_2$ solution. After 5 min, 0.3 mL of 10% $AlCl_3$ solution was added to the above solution. Then, 2 mL of 1 M NaOH solution was added before filling to 10 mL with deionized water. The optical density was then measured at a 510-nm wavelength [25, 26]. The results were expressed as quercetin equivalents (QE) on a dry weight basis [25] (the standard curve equation of quercetin is Abs = 9.6784 × C_{QE} – 0.0327; R = 0.9978).

2.8 Qualitative and quantitative analysis of water-soluble polysaccharides

Polysaccharides were extracted as follows: the powder sample (3 g) was dispersed in 150 mL of distilled water and extracted at 100 °C for 3 h with; the extraction was repeated three times. Then, the solutions were combined, filtered, and concentrated to 50 mL. Next, 96% ethanol was added to the concentrated extract solution to precipitate polysaccharides completely (the ratio of 96% ethanol to extract volume is 4:1) [27, 28].

Qualitative and quantitative analysis: polysaccharides were examined by using the phenol-sulfuric acid colourimetric method with D-glucose as a standard at a wavelength of 490 nm [29]. The standard curve equation of D-glucose is $Y = 0.0082 \times X - 0.0082$, $R = 0.9999$. The content of pure polysaccharides was calculated as follows:

$$\text{Content of pure PS (\%)} = \frac{OD+0.0082}{0.0082} \times V \times \frac{100}{m \times (1-W)} \times \frac{162}{180}$$

where OD is the optical density of the sample; V is the volume of sample; m is the mass of the sample; W is the moisture content of the sample [30].

2.9 Qualitative and quantitative analysis of triterpenoids

The triterpenoid content was determined via the colouring reaction of triterpenoid with vanillin/$HClO_4$ reagent [31]. A 1.0 mL aliquot of the sample in a cuvette was evaporated to remove the solvent, then 0.3 mL of 5% vanillin solution in CH_3COOH and 1 mL of $HClO_4$ were added. The mixture was incubated at 60 °C for 15 min. The mixture was then cooled to 25 °C, and 3.7 mL of acetic acid was added. The optical density was measured at a wavelength of 540 nm against a blank containing the reagent solution without sample. The triterpenoid content was expressed as the number of equivalents of oleanolic acid. The total content of triterpenoid compounds was determined from the calibration curve $Abs = 0.0159 \times C_{AS} + 0.0688$; $R = 0.9993$.

3 Results and discussion

3.1 *In vitro* evaluation of the antioxidant potential of ethanol extract

Total antioxidant capacity

Figure 2 shows that the ethanol extract of *Calocybe indica* has a high total antioxidant activity with the electron transfer mechanism. This antioxidant activity is higher than that of *Ophiocordyceps sobolifera* [32] but lower than that of ascorbic acid.

The study reveals that the antioxidant capacity of the extracts increases with the plant extract concentration, reaching the highest value at 64.94 ± 1.03 mg GA/g or 77.42 ± 0.42 μmol of AS/g at the concentration of 1 mg/mL [14, 22]. This capacity is significantly lower than that of the grape seeds sample (233.2–337.1 μmol of AS/g) [33] and a tea sample (115 mg of GA/g) [22]. However, the total antioxidant capacity of *Calocybe indica*'s extract is higher than that of *Ophiocordyceps sobolifera* (from 5.52 ± 0.14 to 12,71 ± 0.23 mg GA/g or from 3.55 ± 0.15 to 7.87 ± 0.05 μmol AS/g) [32]. This result suggests that this mushroom is a potent antioxidant.

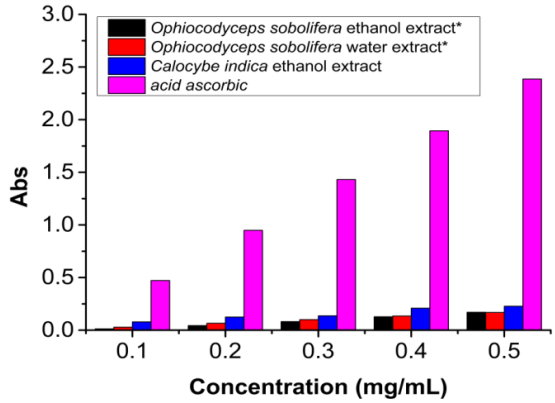

Fig. 2. Antioxidant activity of the ethanol extracts of *Calocybe indica* in total antioxidant capacity model; * Data from [32]

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DPPH radical scavenging activity

Table 1 shows that the DPPH radical scavenging activity of *Calocybe indica*'s ethanol extracts correlates with concentration. The IC_{50} value of this extract is 2.9 times higher than that of *Ophiocordyceps sobolifera* (240.11 against 700 μg/mL) [32] but lower than that of ascorbic acid. It can be seen that IC_{50} of the ethanol extract of *Calocybe indica* is 4.1 times higher than that *Calocybe indica* from India (240.11 vs. 1000 μg/mL) [14]. Thus, the ethanol extract of *Calocybe indica* from Vietnam has a high antioxidant potential.

Table 1. DPPH radical scavenging activity rates of ethanol extract of *Calocybe indica*

Ascorbic acid					
Concentration (μg/mL)	0.8	4	10	20	100
Inhibited DPPH (%)	37.08	88.81	92.40	93.80	96.65
IC_{50} (μg/mL)	1.60				
Ethanol extract of *Calocybe indica*					
Concentration (μg/mL)	100	200	300	400	500
Inhibited DPPH (%)	31.89	44.48	58.24	66.23	72.36
IC_{50} (μg/mL)	240.11				

ABTS radical scavenging activity

The $ABTS^{+\cdot}$ scavenging activity of the ethanol extract of *Calocybe indica* increases with its concentration, namely, 21.24, 30.18, 40.17, 52.06, and 58.76% at 100, 200, 300, 400, and 500 μg/mL, respectively. Its IC_{50} is 398.29 μg/mL. The scavenging rate of this extract is around 60% at 500 μg/mL, much lower than that of ascorbic acid (54.46, 75.85, 86.47, 92.48, and 96.23% at 100, 200, 300, 400, and 500 μg/mL, respectively). At a concentration of 500 μg/mL, the ABTS radical scavenging activity of Vietnam's *Calocybe indica*'s ethanol extract is higher than that of *Calocybe indica* (12.6–22.5%) and *Pleurotus sajor-caju* (13.4–30.2%) from India [14].

3.2 Content of compounds from *Calocybe indica*

Previous studies indicated that the antioxidant potential of medicinal plants was attributed to phenolic and flavonoid compounds [34-36]. According to Mishra et al., phenolic compounds are major contributors to the antioxidant activity of *Calocybe indica* [14]. The content of phenolic and flavonoid compounds in *Calocybe indica* in this study is 29,33 ± 0,16 mg GAE/g and 17,84 ± 0,11 mg QUE/g. This phenolic compound content is equivalent to 45.16% of the antioxidant capacity. In addition, the total phenolic content from *Calocybe indica* in this study is higher than that of *Calocybe indica* from India (14.73–26.72 mgGAE/g) [34]. Furthermore, the total flavonoid content is higher than that of the *Calocybe indica* reported by Ghosh (10.24 ± 3.15 mgQE/g) [15]. It should be noted that *Calocybe indica* from Vietnam is phenol-rich.

According to some researchers [37-40], triterpenoids and polysaccharides make up the valuable biologically active substances in mushrooms. The polysaccharides obtained from *Calocybe indica* have antioxidant and anti-aging activities [17]. The antitumor effect of polysaccharides from the *Calocybe indica* on HeLa cells has also been reported [41]. The content of polysaccharides and triterpenoids in *Calocybe indica* is presented in Table 2.

It is clear that the polysaccharide content is lower than that of *Calocybe indica* from India (the polysaccharides of *Calocybe indica* from India is extracted with cold and hot water and hot NaOH, and the total polysaccharide yield is 8.6%) [42]. Specifically, the total triterpenoid content of *Calocybe indica* has been reported for the first time.

Table 2. Content of compounds from *Calocybe indica* (n = 3)

Sample	Total phenolic (mg GAE/g)	Total flavonoids (mg QUE/g)	Polysaccharides (%)	Total triterpenoids (mg oleanolic acid/g)
Calocybe indica	29.33 ± 0.16	17.84 ± 0.11	5.04 ± 0.04	4.96 ± 0.04

4 Conclusion

The experimental results may provide the theoretical basis for further systematical research and development of mushroom *Calocybe indica*. The antioxidant properties of *Calocybe indica*'s ethanol extract are investigated, and the extract exhibits satisfying activity towards DPPH and ABTS radicals with an IC_{50} value of 240.11 μg/mL and 4398.29 μg/mL. The total antioxidant capacity is equivalent to 64.94 ± 1.03 mg GA/g or 77.42 ± 0.42 μmol AS/g, and the content of polysaccharides is 5.04 ± 0.04%. The content of phenolic and flavonoid compounds is equivalent to 29.33 ± 0.16 mg of GAE/g and 84 ± 0.11 mg of QUE/g, indicating that *Calocybe indica* is a phenol-rich medicinal source. Specifically, the total triterpenoid content of *Calocybe indica* is reported for the first time. Mushroom *Calocybe indica* is a promising resource of natural antioxidants.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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