IN VITRO GROWTH CHARACTERISTICS AND BIOACTIVITIES OF THE CULTIVATED MYCELIUM OF *OPHIOCORDYCEPS SPHECOCEPHALA* FROM VIETNAM

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Abstract. *Ophiocordyceps sphecocephala* is an entomopathogenic fungus that infects wasps. In this report, the *in vitro* growth characteristics of Vietnamese *Ophiocordyceps sphecocephala* collected in Bidoup, Nui Ba, Lam Dong, Vietnam were investigated. The bioactivities of the cultivated mycelium were also studied. The data showed that *O. sphecocephala* had higher yield of mycelium in mushroom complete medium (MCM), malt yeast medium (MY) and Sabouraud dextrose and yeast extract medium (SDY). Yeast extract and silkworm pupa powder were the preferred nitrogen sources. Besides, *O. sphecocephala* could utilize various forms of hexoses such as glucose, fructose, sucrose, lactose, maltose, and starch as the carbon sources. The optimal pH for the growth of *O. sphecocephala* was 6.0. The ethanol extract of *O. sphecocephala* exhibited several bioactivities: the capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, the inhibitory activity against α-glucosidase and the cytotoxicity against MCF-7 (the breast cancer cell line), HeLa (the cervical cancer cell line), Hepa G2 (the liver cancer cell line), Jurkat (the leukemia cell line) and NCI H460 (the lung cancer cell line) with the highest effect on MCF-7, and the lowest effect on NCI H460. Our finding thus demonstrated a potential of employing *O. sphecocephala* mycelium in pharmacology.
Keywords: *radical scavenging, α-glucosidase, cancer cell lines, nitrogen, carbon*

Introduction

Scientific studies have revealed a variety of bioactive compounds, including nucleosides, polysaccharides, ergosterols, *etc*. from many species of *Cordyceps* and allied genera (Isaka et al., 2005; Zhang et al., 2020). These compounds show a wide range of health benefits including immunomodulatory, anti-inflammatory, antioxidant, and anti-tumor effects (Isaka et al., 2005; Zhang et al., 2020). The pharmaceutical benefits of *Cordyceps*, therefore make it interesting for both traditional medicine and modern pharmacology.

In recent years, an increasing number of works have been done on *in vitro* cultivation of *Cordyceps* (Wongsa et al., 2005; Sung et al., 2011a,b; Le et al., 2020; Kontogiannatos et al., 2021; Peng et al., 2023). Successful cultivation of *Cordyceps* offers several

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advantages over the wild harvest, as it enables us to monitor the quality as well as the quantity of the *Cordyceps* products. It also aids in mitigating the effects of over-exploitation on wild populations of *Cordyceps* (Ren and Yao, 2013). In biodiversity conservation, the cultivation of *Cordyceps* allows the preservation and regeneration of the genetic diversity of *Cordyceps* species threatened by climate changes, habitat destruction, over-exploitation and other activities of humans (Ren and Yao, 2013).

Under *in vitro* conditions, many factors influence the growth and production of *Cordyceps* metabolites including genetic factors. Different species of *Cordyceps* have different growth rates and even different strains of the same species also respond differently (Dong and Yao, 2005; Leung and Wu, 2007; Sung et al., 2010; Dang et al., 2018; Tao et al., 2020).

In this study, the *in vitro* growth characteristics of Vietnamese *Ophiocordyceps sphecocephala* collected in Bidoup, Nui Ba, Lam Dong, Vietnam by Mai et al. (2022) were investigated. We also examined the bioactivities of the cultivated *O. sphecocephala* such as the radical scavenging activity, the inhibition activity of α-glucosidase and the cytotoxicity against different cancer cell lines.

Methods

Chemicals

HiMedia (India) chemicals were used for fungal isolation and cultivation. All other chemicals and reagents, unless otherwise noted, were purchased from Sigma Aldrich (USA) and Merck (Germany).

Fungal isolation

The fruiting body of *O. sphecocephala* was soaked in 70% (v/v) ethanol for 1 min, then sliced to release spores. The spores were then suspended in 0.5% (w/v) water agar and plated on potato dextrose agar (PDA) supplemented with 100 μg/ml streptomycin. The PDA plates were then incubated at 20ºC for 4 weeks. For molecular identification of the isolates, D1-D2 and ITS regions were amplified and aligned with the published D1-D2 and ITS sequences of Vietnamese *O. sphecocephala* (MT235760 and MW684021, respectively) (Mai et al., 2022).

The broth culture of *O. sphecocephala* was prepared by transferring two agar blocks (0.5 × 0.5 cm) of mycelium into a 250 ml flask containing 100 ml of mushroom complete medium (MCM), at pH 6.0. The flasks were incubated in a shaker at 20ºC and 200 rpm for 3 weeks and used in further experiments.

To study the effect of pH on the growth of *O. sphecocephala*, 5 ml of the broth culture was inoculated in 500 ml flasks containing 100 ml of MCM with various levels of initial pH ranging from 3.0 to 8.0 at the interval of 1.0.

To analyze the effect of medium on the growth of *O. sphecocephala*, 5 ml of the broth culture was inoculated in 100 ml liquid media of potato dextrose (PD), Hennerberg medium (HEN), Sabouraud dextrose and yeast extract (SDY), malt yeast (MY) and mushroom complete medium (MCM) at pH 6.0 (*Table 1*).

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Table 1. *The ingredients of the culture media (Lee et al., 2013)*

Medium	Organic components (g/l)						Inorganic components (g/l)
	Glucose	Maltose	Potato starch	Peptone	Malt extract	Yeast extract	
PD	20		4				
HEN	50						2.0 $NaNO_3$, 0.5 $MgSO_4$, 0.1 $CaCl_2$, 1.0 K_2HPO_4 2.0 KNO_3
SDY	40			10		10	
MY	4				10	4	
MCM	20			2		2	1.0 K_2HPO_4, 0.5 $MgSO_4$ 0.5 KH_2PO_4

The effect of nitrogen source on the growth of *O. sphecocephala* was investigated by inoculating 5 ml of the broth culture in 100 ml of MCM, pH 6.0. The nitrogen source of MCM was substituted with 4 g/l of one of the following: yeast extract, peptone, malt extract, tryptone, or silkworm pupa powder.

To study the effect of carbon source on the growth of *O. sphecocephala*, 5 ml of the broth culture was inoculated in 100 ml of MCM, pH 6.0 by substituting the carbon source with 20 g/l of one of the following: glucose, fructose, xylose, maltose, sucrose, lactose, or starch.

All the inoculations were incubated at 20ºC and 200 rpm for 28 days. Each experiment was repeated in triplicate.

Fungal extraction

The extraction of *O. sphecocephala* was conducted using mycelium harvested from the medium containing 20 g/l glucose, 4.0 g/l yeast extract, 1.0 g/l K_2HPO_4, 0.5 g/l $MgSO_4$ and 0.5 g/l KH_2PO_4. Five grams of dried powder of *O. sphecocephala* mycelium was extracted in 100 ml of 50% (v/v) ethanol. In each extraction, the solution was sonicated for 6 mins at full power in two intervals of 3 mins using an ultrasonic bath (Elmasonic S30H, Elma, Germany). The solution was then left stirring at 40°C for 24 h. The filtrate was harvested, dried at 40°C and stored at 4°C for further analysis.

Bioactivities of the cultivated mycelium

DPPH radical scavenging assay

The free radical scavenging ability of the extract was determined according to the method of Herald et al. (2012). In a 96-well plate, 200 µl of 150 µM DPPH in 80% (v/v) methanol and 25 µl of extract at different concentrations was added. 100 µg/ml trolox and 80% (v/v) methanol were used as the positive controls and the negative control, respectively. The absorbance of the mixtures at 517 nm was recorded using a 96-well micro-titer plate reader (Synergy HT, Biotek Instruments). The percentage of DPPH free radical neutralization was calculated using the formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{OD negative control} - \text{OD test}}{\text{OD negative control}} \times 100\% \quad \text{(Eq.1)}$$

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The inhibitory concentration (IC_{50}) value was determined based on the response curve of % DPPH radical scavenging activity and the extract concentrations.

α-glucosidase inhibition activity

The inhibitory activity of the extracts on α-glucosidase enzyme was evaluated as described by Wan et al. (2013). In a 96-well plate, 20 µl of α-glucosidase 1.0 unit/ml in phosphate buffer, pH 6.8 and 125 µl of extract at different concentrations was added. 1.0 mg/ml acarbose and water were used as the positive control and the negative control, respectively. The mixtures were incubated at 37°C for 15 mins. Subsequently, 20 µl of 5 mM p-nitrophenyl-α-D glucopyranoside (pNPG) was added into the reactions and incubated for a further 30 mins. The reactions were stopped by adding 80 µL of 0.2 M Na_2CO_3. The absorbance of p-nitrophenol produced in the mixtures at 405 nm was recorded using a 96-well micro-titer plate reader (Synergy HT, Biotek Instruments).

The percentage of α-glucosidase inhibitory activity was calculated using the formula:

$$\text{Inhibitory activity (\%)} = \frac{\text{OD negative control} - \text{OD test}}{\text{OD negative control}} \times 100\% \qquad \text{(Eq.2)}$$

The inhibitory concentration (IC_{50}) value was determined based on the response curve of α-glucosidase inhibitory activity and the extract concentrations. All experiments were conducted in triplicates.

Cytotoxicity activity

The cytotoxicity activity of the extract was determined using the method described by Nguyen and Ho-Huynh (2016). Hepa G2 (the liver cancer cell line), NCI H460 (the lung cancer cell line), Jurkat (the leukemia cell line), MCF-7 (the breast cancer cell line) and HeLa (the cervical cancer cell line) were procured from the American Type Culture Collection (Manassas, Rockville) and grown in either Eagle's Minimal Essential Medium (EMEM) (for Hepa G2, NCI H460, MCF-7 and HeLa cell lines) or Roswell Park Memorial Institute medium (RPMI) (for Jurkat) supplemented with 2 mM L-glutamine, 20 mM HEPES, 0.025 µg/ml amphotericin B, 100 UI/ml penicillin G, 100 µg/ml streptomycin and 10% (v/v) of fetal bovine serum.

All cells were cultured in 96 well-plates at 37°C in 95% air and 5% CO_2 humidified incubators. The cell density cultured in each well was 10^4 cells/ well for Hepa G2, MCF-7 and HeLa; $7.5.10^3$ cells/ well for NCI H460 and 5.10^4 cells/well for Jurkat. After 24-hour incubation, the cells were treated with *O. sphecocephala* extracts at different concentrations for 48 hours. Camptothecin (Calbiochem) was used as the positive control at the concentrations of 0.07 µg/ml (Hepa G2), 0.007 µg/ml (NCI H460), 0.005 µg/ml (Jurkat), 0.01 µg/ml (MCF-7) and 1.0 µg/ml (HeLa). Water was used as the negative control.

After 48 hours of incubation, sulforhodamine B staining assays on the tested cell lines were conducted. Cells were fixed with cold trichloroacetic acid (50% for Jurkat and 70% for other cell lines), stained with fluorescent dye 0.2% sulforhodamine B for 20 mins, then washed with 1% (v/v) acetic acid. The protein-bound dye was then dissolved in 10 mM Tris-base solution (Promega). Optical density values at 492 nm and 620 nm were determined with a 96-well micro-titer plate reader (Synergy HT, Biotek Instruments).

The cytotoxicity activity was calculated using the formular:

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$$\text{Toxicity activity (\%)} = \frac{\text{OD negative control} - \text{OD test}}{\text{OD negative control}} \times 100\% \quad \text{(Eq.3)}$$

The inhibitory concentration (IC_{50}) value was determined based on the response curve of cytotoxicity activity and the extract concentrations. All experiments were conducted in triplicates. Furthermore, four cell lines, except Jurkat were also examined under an inverted microscope (CKX41 Olympus) at 480 times magnification.

Statistical analysis

All data were analyzed in SPSS 20 (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) followed by Tukey's test at P-value < 0.05. The data that do not share a superscript letter are significantly different at $p < 0.05$ by one-way ANOVA.

Results and discussion

Numerous species of *Cordyceps* have been isolated and cultivated *in vitro*, many of them have fast growth as seen in *C. militaris, C. bassiana, O. opentatomae*; whereas *O. sinensis, C. martial, C. rosea, O. heteropoda, etc*. have a slow rate of mycelium growth (Sung et al., 2010). It is also observed that the slowly growing species of *Cordyceps* thrive faster in submerged conditions than in solid medium, therefore submerged culture is an ideal cultivation technique for species of *Cordyceps* with slow growth rates (Dong and Yao, 2005; Mei et al., 2013; Kaushik et al., 2019). Our experiments showed that *O. sphecocephala* had a slow growth rate in solid medium, only 3.0 to 3.5 cm of colony diameter on PDA was obtained after 4 weeks of incubation. We thus used submerged cultivations for all other experiments.

Effect of medium on mycelial growth of O. sphecocephala

Among the media tested, higher mycelial yield of *O. sphecocephala* was recorded in the medium containing organic nitrogen MCM, MY and SDY. Whereas PD and HEN without containing organic nitrogen produced lower yield of mycelium (*Fig. 1*). The result therefore indicated that the presence of organic nitrogen was essential for the growth of *O. sphecocephala* mycelium. Research has also revealed that inorganic nitrogen sources have a minor influence on the growth of *C. militaris* and *O. sinensis* (Dong and Yao, 2005; Lee et al., 2013). Similarly, *Ophiocordyceps heteropoda* and *O. longissima* exhibit greater growth on the medium supplemented with organic nitrogen such as Hamada and MYA than on the nitrogen deficient media such as Czapek-Dox (Sung et al., 2011a,b). Organic nitrogen containing media such as MCM, SDAY and MYA are also the preferred media for *C. militaris* (Shrestha et al., 2006; Lee et al., 2013). Likewise, MCM is the preferred medium for *C. cardinalis* (Sung et al., 2010).

And although SDY medium had a five-fold higher nitrogen concentration in comparison to MCM medium, the mycelium yield was similar in both media (*Table 1*, *Fig. 1*). The studies on *O. sinensis* show that high nitrogen content in culture media suppresses spore germination and mycelium growth (Mei et al., 2013; Kaushik et al., 2019, 2020). The ability of *O. sphecocephala* to grow in low concentration of nitrogen may help optimize costs in the mass production of *O. sphecocephala* mycelium, since MCM media is generally cheaper than SDY media.

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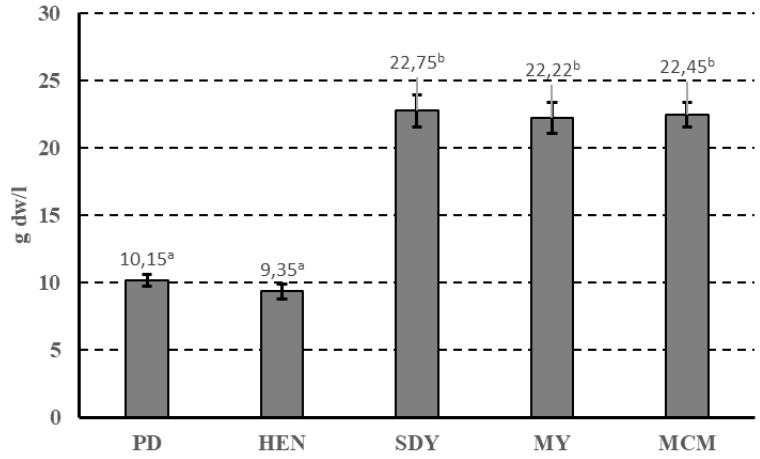

Figure 1. *Effect of medium on the growth of O. sphecocephala. Error bars indicate 95% confident limits. The data that do not share a superscript letter are significantly different at p<0.05 by one-way ANOVA*

Our preliminary experiments on the fruiting body formation of *O. sphecocephala* using showed that the stromata on PD and HEN media were long and fibrous, whereas thicker stromata were obtained in MCM, SDY and MY (*Fig. 2*). Additional experiments are being carried out in our laboratory to elucidate the effect of the cultivation media on the formation of the fruiting bodies.

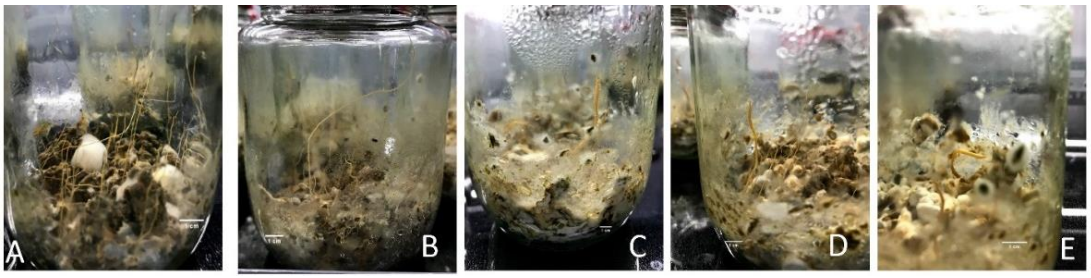

Figure 2. *Fruiting body formation in A. PD, B. HEN, C. MCM, D. SDY, E. MY after 5 months of cultivation. Bars: 1.0 cm*

Effect of nitrogen source on mycelial growth of O. sphecocephala

Our data showed that *O. sphecocephala* preferred yeast extract and silkworm pupa powder over other nitrogen sources such as peptone, malt extract or tryptone. No significant difference in the mycelial yield was observed when using peptone, malt extract and tryptone as the nitrogen source (*Fig. 3*). Yeast extract and silkworm pupa have been widely used in the cultivation of *C. militaris* (Hong et al., 2010; Yang et al., 2014; Kato et al., 2021). Differently, *C. cardinalis* and *O. sinensis* display a preference for peptone as a nitrogen source (Dong and Yao, 2005; Sung et al., 2010). Peptone and tryptone are the appropriate nitrogen sources for the growth of *C. militaris* (Lee et al., 2013; Raethong et al., 2020).

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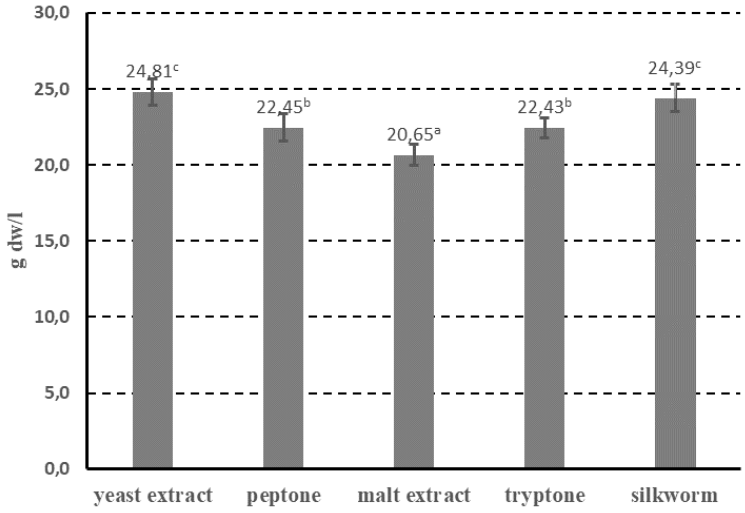

Figure 3. *Effect of nitrogen source on the growth of O. sphecocephala. Error bars indicate 95% confident limits. The data that do not share a superscript letter are significantly different at p<0.05 by one-way ANOVA*

Effect of carbon source on mycelial growth of O. sphecocephala

Among seven carbon sources tested, *O. sphecocephala* exhibited the lowest mycelial yield when using xylose. The mycelial yield from the other tested carbon sources was statistically comparable (*Fig. 4*). The results therefore indicated that *O. sphecocephala* had a higher capacity to employ hexose sugars in various combinations of polymers and isomers than using pentose sugar. Earlier research in *C. militaris* also demonstrates a preference for glucose, fructose, mannitol, and sucrose as the carbon source over xylose and arabinose (Lee et al., 2013; Dang et al., 2018; Raethong et al., 2020). It is postulated that the energy expenditure of the cells may be increased when taking pentose instead of hexose (Raethong et al., 2020). Many other species of Cordyceps also display a preference for hexose such as *C. cardinalis* prefers maltose (Sung et al., 2010); mannitol, maltose and sucrose promote the growth of *O. sinensis* (Dong and Yao, 2005).

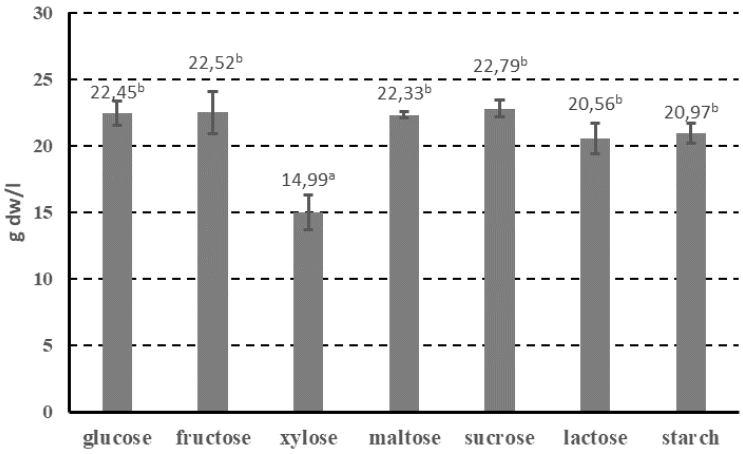

Figure 4. *Effect of carbon source on the growth of O. sphecocephala. Error bars indicate 95% confident limits. The data that do not share a superscript letter are significantly different at p < 0.05 by one-way ANOVA*

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Not only important for the growth rate of *Cordyceps*, the nitrogen and carbon compositions in the growing medium also have a significant influence on the production of bioactive compounds in *Cordyceps* (Dong and Yao, 2005; Mei et al., 2013; Kaushik et al., 2019, 2020; Li et al., 2020; Tao et al., 2020).

Effect of pH on mycelial growth of O. sphecocephala

The pH value of the cultivation media has a substantial effect on the growth of *Cordyceps*. Our data showed that *O. sphecocephala* could not survive at pH 3.0 and 4.0 (*Fig. 5*). And the optimal pH for the growth of *O. sphecocephala* mycelia was 6.0 (*Fig. 5*). *C. militaris* displays fast growth at both pH 6.0 to 7.0 (Shrestha et al., 2006; Lee et al., 2013) while *C. cardinalis*, *O. heteropoda* and *O. longissima* achieve optimal growth at pH 7.0 (Kim et al., 2010; Sung et al., 2010, 2011a,b).

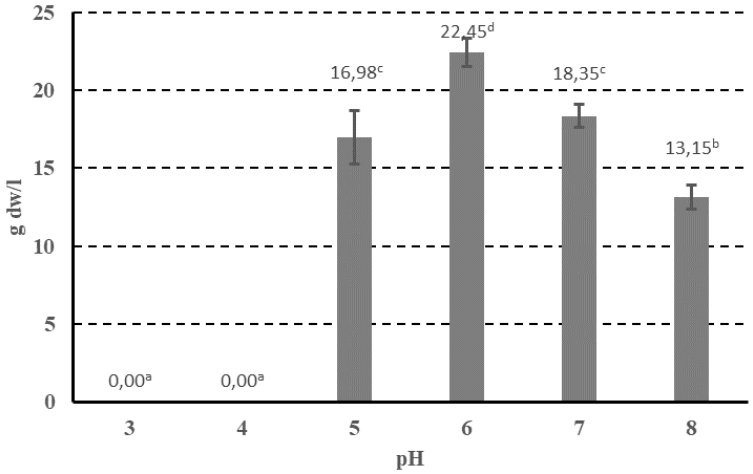

Figure 5. *Effect of pH on the growth of O. sphecocephala. Error bars indicate 95% confident limits. The data that do not share a superscript letter are significantly different at $p < 0.05$ by one-way ANOVA*

Bioactivities of the cultivated mycelium

The research by Kornsakulkarn et al. (2018) has identified ten β-carboline alkaloids and four isocoumarins using ethyl acetate extract of Thai *O. sphecocephala* BCC 2661. Among those detected, sphecolines A and sphecolines C show cytotoxic activity against human small-cell lung cancer NCI H187. However, many species of *Cordyceps* such as *O. sinensis*, *C. militaris*, *C. cicadae* and *O. xuefengensis* are traditionally used as a tonic prepared by steeping the fruiting bodies in local-made alcohol or hot water (Panda and Swain, 2011; Wang et al., 2012; Jin et al., 2018). In Vietnam, people also collect *O. sphecocephala* and use the fruiting bodies to prepare tinctures.

Accordingly, it is worthwhile to investigate the bioactivity of the ethanol: water (50:50, v/v) extract of *O. sphecocephala*.

Free radical scavenging ability

Respiration and other activities such as immune responses produce a lot of free radicals. Antioxidant molecules neutralize free radicals by donating their electrons, thus minimize biological damage caused by free radicals (Auten and Davis, 2009). Cancer

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cells have higher levels of free radicals in their cells compared to normal cells. Hence, antioxidants are considered to be beneficial both for the prevention and treatment of cancers (Nakamura and Takada, 2021).

All the tested concentrations of *O. sphecocephala* exhibited the capacity to scavenge DPPH radical; the IC_{50} value was calculated at the concentration of 93.4 ± 3.64 µg/ml (*Fig. 6*). The antioxidant activities are also detected in many other *Cordyceps* species such as *C. militaris, O. formosana*, *O. xuefengensis* and *O. sobolifera* (Li et al., 2017; Dang et al., 2018; Qin et al., 2018; Quynh, 2022; Thi et al., 2022). The antioxidant property of *O. sphecocephala* suggests that this fungus could be used in the treatment of diseases caused by free radicals.

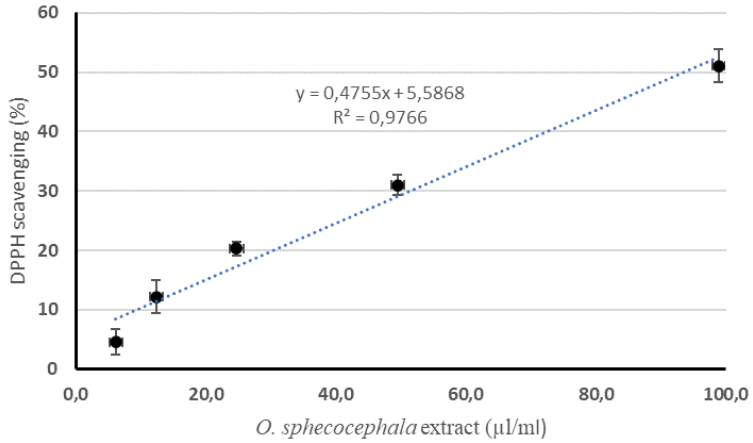

Figure 6. *Scavenging activity to DPPH of O. sphecocephala. Error bars indicate 95% confident limits*

α-glucosidase inhibition activity

α-glucosidase is an enzyme in the intestinal tract that is responsible for the hydrolytic conversion of complex carbohydrates into monosaccharides (Indrianingsih and Tachibana, 2017). The mono sugars then enter the bloodstream, and this can lead to a sharp rise in blood sugar in patients with diabetes. α-glucosidase inhibitors slow down the production of mono sugars and delay the influx of glucose into the bloodstream (Indrianingsih and Tachibana, 2017).

Our results showed that the extract of *O. sphecocephala* at the concentrations from 17.25 to 276 µg/ml could inhibit the activity of α-glucosidase (*Fig. 7*). It is therefore suggested the possibility of utilizing *O. sphecocephala* for assisting diabetic care. Because the inhibitory activity was maximum at only 43.57± 4.5%, the IC50 value of the extract against α-glucosidase could not be determined. The inhibitory activity against α-glucosidase is also reported in *C. militaris* and *O. sinensis* (Hang et al., 2020; Wu et al., 2020).

Cytotoxicity activity

The extract of *O. sphecocephala* was toxic to all five cancer cell lines tested (*Fig. 8*). The strongest effect of cytotoxicity was observed in the MCF-7 cell line, followed by the group of HeLa and Jurkat cell lines (*Fig. 8*). The effect of our extract was lowest in lung cancer cell line (NCI H460) (*Fig. 8*). Among ten β-carboline alkaloids and four

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isocoumarins in the ethyl acetate extract of Thai *O. sphecocephala*, only sphecolines A and sphecolines C affect the lung cancer cell line (Kornsakulkarn et al., 2018). This difference thus postulated two possible explanations: first, the Thai and Vietnamese *O. sphecocephala* produce different bioactive compounds; second, the extracted bioactive compounds when using ethyl acetate as the extraction solvent are different from those when using 50% ethanol as the extraction solvent. Further experiments are going on in our laboratory to evaluate these possibilities.

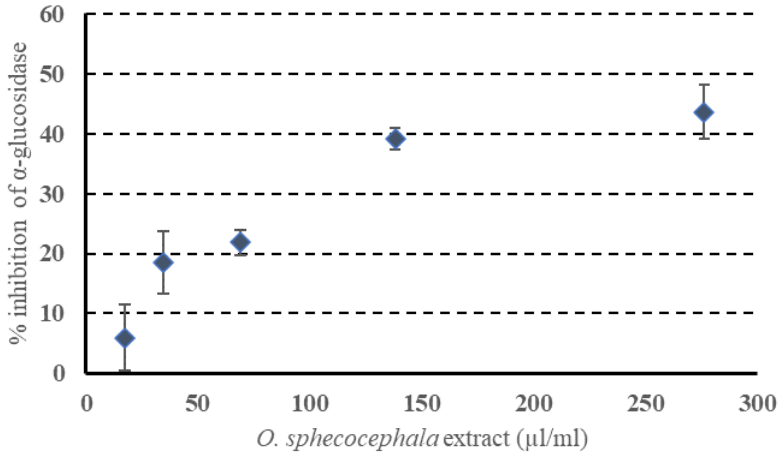

Figure 7. *α-glucosidase inhibition activity of O. sphecocephala. Error bars indicate 95% confident limits*

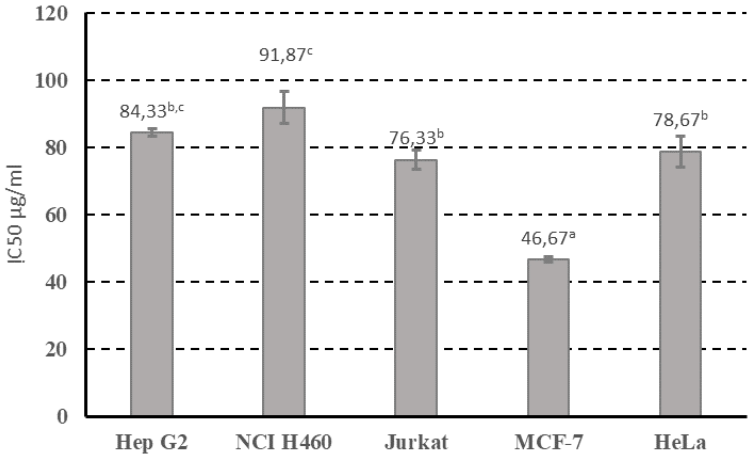

Figure 8. *Cytotoxicity of O. sphecocephala extract against different cancer cell lines. Error bars indicate 95% confident limits. The data that do not share a superscript letter are significantly different at $p < 0.05$ by one-way ANOVA*

It was also observed that the extract of *O. sphecocephala* caused severe changes in the morphology of cells including shrinkage and fragmentation of the cells, which resulted in a significant decrease in the cell density and eventually led to cell death (*Fig. 9*). It was also observed that MCF-7 cell lines exhibited more morphological damage in comparison to other cell lines (*Fig. 9*).

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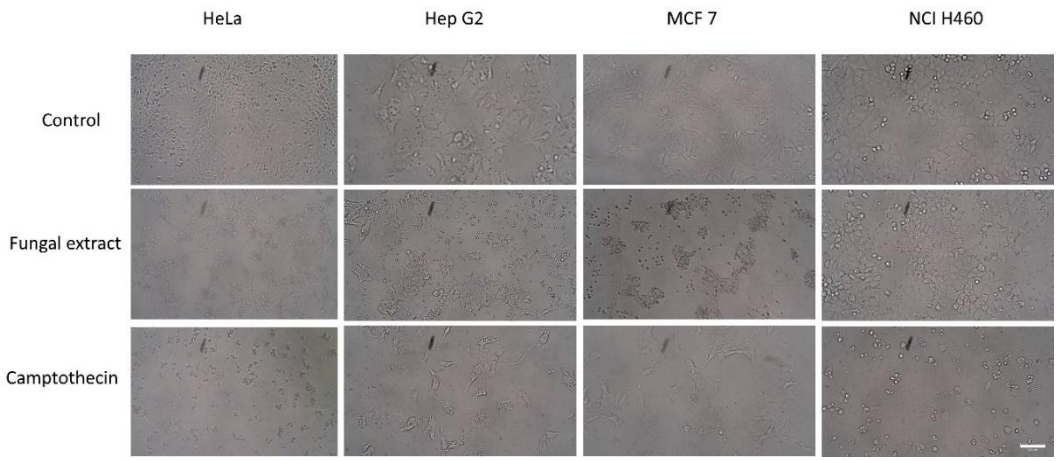

Figure 9. *Cancer cell lines in water (negative control), 40 µg/ml of O. sphecocephala extract and camptothecin (positive control). Bar 300 µm*

Conclusions

Our data showed that *O. sphecocephala* produced the highest mycelial yield in MCM, MY and SDY. This fungus also preferred to use yeast extract as well as silkworm pupa powder as nitrogen sources. The preferred carbon sources were various forms of hexose such as glucose, fructose, sucrose, lactose, maltose, and starch. The optimal pH for the growth of *O. sphecocephala* was 6.0 and no growth was recorded at pH 3.0 - 4.0. The ethanol extract of *O. sphecocephala* exhibited several bioactivities: the capacity to scavenge DPPH radical; the inhibitory activity against α-glucosidase; the cytotoxicity against MCF-7, HeLa, Hep G2, Jurkat and NCI H460 with the strongest effect was on MCF-7 (the breast cancer cell line), and the lowest effect was on NCI H460 (the lung cancer cell line). The results therefore proposed potential uses of the cultivated mycelium of *O. sphecocephala* in cancer and diabetic treatments.

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Conflicts of Interests. The authors declare that they have no conflict of interests.

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