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**Research Article**

# IMPACT OF CULTURE MEDIA AND DURATION ON ANTIOXIDANT AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES OF METHANOL EXTRACT FROM *Graphis handelii* MYCOBIONT

***Nguyen Thanh Dat[1], Nguyen Hong Linh[1],***
***Pham Truong Phu Van Tai[1], Vo Thi Phi Giao[2], Tran Thi Minh Dinh[1*]***
*[1]Ho Chi Minh City University of Education, Vietnam*
*[2]University of Science, Vietnam National University Ho Chi Minh City, Vietnam*
*[*]Corresponding author: Tran Thi Minh Dinh – Email: dinhttm@hcmue.edu.vn*
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**ABSTRACT**

*This study investigated the influence of culture media and culture time on the antioxidant and α-glucosidase inhibitory activities of the methanol extract obtained from the mycobiont of Graphis handelii. Different culture media, including MIX, PDA, and SAB4%, were employed to culture the mycobiont, and the biomass was harvested during different culture periods (4, 8, and 12 weeks). The resulting methanol extracts were evaluated for their free radical scavenging and alpha-glucosidase inhibitory activities. The results showed that all nine methanol extracts exhibited antioxidant capacity through DPPH scavenging in vitro assay. Among them, the extract derived from the mycobiont cultured in MIX medium for four weeks had the most vigorous activity, scavenging 86.52% DPPH at the concentration of 200 µg/mL, and its $IC_{50}$ value was determined to be 107.10 ± 5.39 µg/mL. In contrast, all extracts showed weak or no α-glucosidase inhibitory activities. The findings showed that the culture medium composition impacts the production of antioxidant compounds in the mycobiont. This research holds significance for optimising cultivation conditions to maximise the biological potential of Graphis handelii mycobiont extracts, particularly for antioxidant activities.*

***Keywords:*** antioxidant; alpha-glucosidase inhibition; *Graphis handelii;* lichen mycobiont

## 1. Introduction

Lichens are a symbiotic association between fungi and unicellular algae, filamentous algae, or cyanobacteria. Up to 98% of the fungi that form lichens are Ascomycetes, and the rest are Basidiomycetes. About 30,000 species of lichens have been found living in many different environments, covering 6-10% of the Earth's surface (Nash, 2008). Lichens exhibit

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significant diversity in their physical structure and are conventionally categorised into three primary morphological groups: crustose, foliose, and fruticose (Asplund & Wardle, 2017).

Nowadays, lichens are considered a potential source of medicinal materials as more and more compounds are found to have potential applications in treatment. In recent studies, scientists have suggested that the origin of these active compounds is the secondary metabolites of lichen-symbiotic fungi (Karagöz & Öztürk Karagöz, 2022). In 2020, over 1,000 secondary metabolites were obtained from lichens in research projects. Among them, many compounds such as depsides, depsidones, dibenzofurans, quinones, chromones, carotenoids, polysaccharides, monosaccharides, fatty acids... were found only in lichens (Goga et al., 2020). Besides, experiments revealed specific nutritional demands for lichen mycobiont growth and substance production (Behera et al., 2006). However, studies on the effects of culture medium and culture time on producing secondary metabolites in lichens are still scarce.

As of March 2024, few research studies have reported the biological activities of compounds and crude extracts of the *G. handelii* (Tran et al., 2024). The authors reported on α-glucosidase enzyme inhibition, HIV-1 reverse transcriptase inhibition, SARS-CoV-2 main protease ($M^{pro}$) inhibition, anti-inflammatory activity, and cytotoxicity against several cancer cell lines. However, no studies have investigated the effects of culture medium and culture time on their activities.

In this paper, we report for the first time the effects of culture medium and incubation time on the antioxidant and α-glucosidase inhibitory activities of the methanolic extract from the mycobiont of the lichen *G. handelii*.

## 2. Materials and methods

### *2.1. Materials*

The lichen mycobiont UE-MY05, obtained from a *G. handelii* sample (Figure 1) collected in Kien Tuong, Long An (10.766815, 105.940536) in May 2022, was used in this research. The lichen sample was identified to be Graphis handelii by Dr. Vo Thi Phi Giao, Faculty of Biology and Biotechnology, University of Sciences, Ho Chi Minh City, Viet Nam. The mycobiont belongs to the lichen mycobiont collection maintained by Dr. Tran Thi Minh Dinh at the Department of Biology, Ho Chi Minh City University of Education.

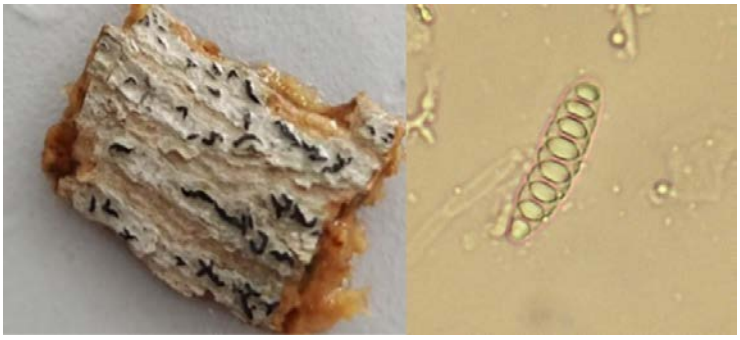

***Figure 1.*** *The Graphis handelii lichen sample used in this study and its ascospore*

### *2.2. Chemicals*

Chemicals used in this study include methanol (Merck, Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (TCI, Japan), dimethyl sulfoxide (DMSO) (Sigma Aldrich, UK), disodium carbonate (AR, China), sodium dihydrogen phosphate phosphate (BioBasic, Canada), disodium hydrogen phosphate (BioBasic, Canada), *p*-nitrophenyl-α-D-glucopyranoside (pNPG) (Sigma Aldrich, UK), α-glucosidase from *Saccharomyces cerevisiae* (Sigma Aldrich, UK).

### *2.3. Methods*

*2.3.1. Mycobiont culture*

A small mycobiont fragment, approximately 1 x 1 mm in size, was aseptically transferred onto the surface of media in Petri disks, including MIX medium (8 g peptone from meat, 8 g peptone from casein, 20 g malt extract, 5 g sodium chloride, 4 g glucose, 15 g agar, and 1 L distilled water), SAB4% medium (40 g sucrose, 10 g peptone, 15 g agar, and 1 L distilled water), and PDA medium (300 g potato, 50 g sucrose, 15 g agar and 1 L distilled water) (Upreti et al., 2015). The disks were incubated in the dark at 18 ± 2ºC. Mycobiont colonies were harvested after 4, 8, and 12 weeks of incubation.

*2.3.2. Methanol extract*

The methanol extract was prepared based on the published literature with slight modifications (Tran et al., 2024). The mycobiont colonies were dried in an oven at 50°C until completely dry. The dry biomass was then ground and soaked in methanol solvent at a ratio of 1:1, repeated ten times. After the soaking stage, the mixture was filtered through filter paper to remove the biomass. The filtrate was evaporated from the solvent using a rotary evaporator to obtain the methanol extract. The extract was left at room temperature to evaporate all the solvent for about seven days. The extraction efficiency was calculated as described in a previous publication (Tran et al., 2023).

*2.3.3. DPPH scavenging assay*

The antioxidant activity of the methanol extract of *G. handelii* mycobiont was determined using the DPPH free radical scavenging assay following the protocol described in the published literature (Kosanić et al., 2011). DPPH solution (0.08 mM) and extracts (200 µg/mL) were prepared in methanol solvent. A 200 µL aliquot of each sample and 1 mL DPPH solution were added to an Eppendorf tube. For the negative control, the methanol extract was replaced with methanol solvent. The mixture was incubated in the dark at room temperature for 30 min. After incubation, the sample's optical density (OD) was measured at a wavelength of 517 nm. The samples with the highest activity were analysed at different concentrations around the $IC_{50}$ value. The free radical scavenging rate was determined using the following formula:

$$\textbf{DPPH scavenging percentage (\%)} = \frac{OD\ of\ control - OD\ of\ sample}{OD\ of\ control} \times 100$$

*2.3.4. α-Glucosidase inhibitory assay*

The method for investigating the α-glucosidase enzyme inhibitory activity was based on the literature (Do et al., 2022). Briefly, methanolic extract was diluted in 0.1 mM phosphate buffer, pH 6.9, containing 5% DMSO. α-Glucosidase enzyme (0.1 U/mL) and the substrate p-nitrophenyl-α-D-glucopyranoside (5 mM) were prepared in 0.1 mM phosphate buffer pH 6.9. In a 96-well plate, 50 µL of extract (in triplicate) was added to each well, followed by 40 µL of the enzyme solution. The methanolic extract was replaced in three control wells with a 5% DMSO solution. The plate was incubated for 20 min at 37°C. Then, 40 µL of substrate solution was added to each well, and the incubation continued for another 20 min at 37°C. Next, 130 µL of 0.2 M $Na_2CO_3$ was added to the mixture to stop the reaction. The absorbance of the mixture was measured at 405 nm using a multimode microplate reader (CLARIOstar plus, BMG LABTECH, Ortenberg, Germany). The percentage of inhibition of α-glucosidase activity was evaluated using the following formula:

$$\textbf{α-Glucosidase inhibition percentage (\%)} = \left(1 - \frac{OD\ of\ sample}{OD\ of\ control}\right) \textbf{x 100}$$

*2.3.5. Data analysis*

The experiments were carried out in triplicates. The obtained results were analysed using MS Excel 2016. Data were presented in this paper as mean ± standard deviation. The graphs were generated using GraphPad Prism 8 (USA).

## 3. Results and discussion

### *3.1. Mycobiont culture and methanol extract*

The *G. handelii* mycobiont (Figure 2) was cultured on three different media: SAB4%, PDA, and MIX, at 18 ± 2°C. After incubation periods of 4, 8, and 12 weeks, the mycobiont colonies were harvested, separated from the culture media, and dried in an oven at 50°C. The dried mycobiont biomass was then ground into a fine powder and used to prepare a methanol extract. The data for dry mycobiont mass, crude methanol extract yield, and extraction efficiency are presented in Table 1.

***Table 1.*** *Dry mycobiont mass, crude methanol extract yield, and extraction efficiency*

| | **Medium** | **Culture period (weeks)** | | |
|---|---|---|---|---|
| | | **4** | **8** | **12** |
| **Dried biomass (mg)** | PDA | 1,629.40 | 3,553.10 | 9,828.00 |
| | SAB4% | 1,583.80 | 4,118.40 | 6,233.70 |
| | MIX | 1,305.90 | 3,518.90 | 7,105.80 |
| **Methanol extract (mg)** | PDA | 149.00 | 401.40 | 716.10 |
| | SAB4% | 305.20 | 625.20 | 382.40 |
| | MIX | 77.40 | 557.50 | 1068.90 |
| **Extraction efficiency (%)** | PDA | 9.14 | 11.30 | 7.29 |
| | SAB4% | 19.27 | 15.18 | 6.13 |
| | MIX | 5.93 | 15.84 | 15.04 |

As shown in Table 1, the crude methanol extract yield varied depending on the culture medium and cultivation time. This can be explained by mycobionts producing different

compounds under different nutrient conditions and cultivation times. However, overall, the extraction efficiency of *G. handelii* was relatively low in all the media and cultivation times studied. All of the nine resulting methanolic extracts were used to evaluate antioxidant and α-glucosidase inhibitory activities.

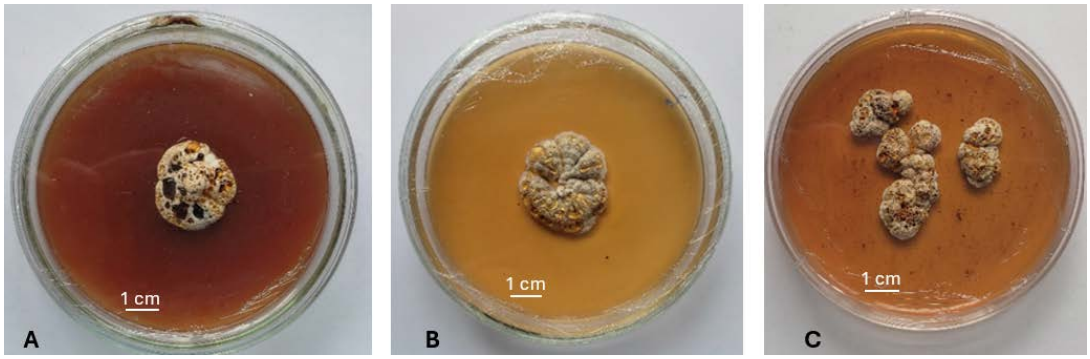


***Figure 2.*** *G. handelii mycobiont after 12 weeks of culture*
A: on MIX medium, B: on SAB4% medium, C: on PDA medium

### *3.2. DPPH scavenging activity*

The results of the antioxidant activity assay of mycobiont methanolic extracts at a final concentration of 200 µg/mL are presented in Table 1.

***Table 2.*** *DPPH scavenging percentage of mycobiont extracts at the concentration of 200 µg/mL*

| No | Medium | Time of culture (weeks) | | |
|---|---|---|---|---|
| | | 4 | 8 | 12 |
| **1** | **PDA** | 72.00 | 32.40 | 51.52 |
| **2** | **SAB4%** | 32.59 | 67.78 | 82.27 |
| **3** | **MIX** | 86.52 | 71.19 | 62.51 |

As shown in Table 2, DPPH scavenging activity of methanolic extracts depended on both the culture medium and cultivation time. In MIX medium, the DPPH radical scavenging activity of the extracts exhibited an inverse relationship with culture time. The highest activity was observed after 4 weeks, followed by a gradual decrease at 8 and 12 weeks. Conversely, SAB4% medium showed increasing DPPH radical scavenging activity with culture time. The methanolic extract obtained from *G. handelii* mycobiont cultured in SAB4% displayed the weakest activity at four weeks but gradually increased at 8 and 12 weeks. Interestingly, in the PDA medium, the DPPH radical scavenging activity of the methanolic extract from the *G. handelii* mycobiont was 2.52 times higher at 12 weeks compared to 4 weeks. In contrast, the methanolic extract from PDA-cultured *G. handelii* mycobiont exhibited fluctuating activity throughout the culture period. It peaked at four weeks, then dropped to its lowest point at eight weeks before showing a slight increase at 12 weeks.

During the same culture period, the DPPH radical scavenging activity of the methanolic extracts from the mycobiont biomass cultured on different media differed. When the mycobiont was harvested at four weeks, the methanolic extract from the mycobiont

biomass cultured in MIX medium showed the strongest DPPH scavenging activity (86.52%), which was 2.65 times higher than the activity of the methanolic extract from the biomass cultured in SAB4% medium, which had the weakest activity. At the 8-week harvest time, the activity of the methanolic extract from the mycobiont biomass cultured in MIX medium still showed the strongest activity; however, the weakest DPPH radical scavenging activity was that of the methanolic extract from the mycobiont biomass cultured in PDA medium, and the difference between the strongest and weakest activities was 2.22 times. At 12 weeks, the DPPH radical scavenging activity of the methanolic extract from the mycobiont biomass cultured in SAB4% medium showed the strongest activity (82.27%), and the methanolic extracts from the mycobiont biomass cultured in PDA and MIX media showed weaker activities. However, their activities were all >50%, and the difference in activity between the extract with the strongest activity and the extract with the weakest activity was only 1.60 times.

Based on the above results, the three methanolic extracts with the highest DPPH scavenging percentages were diluted to different concentrations using a two-fold dilution method and their antioxidant capacity was evaluated. The correlation curves between extract concentration and DPPH scavenging percentage were constructed using GraphPad Prism 8.0.1 software to determine the $EC_{50}$ values. The results are presented in Figure 1.

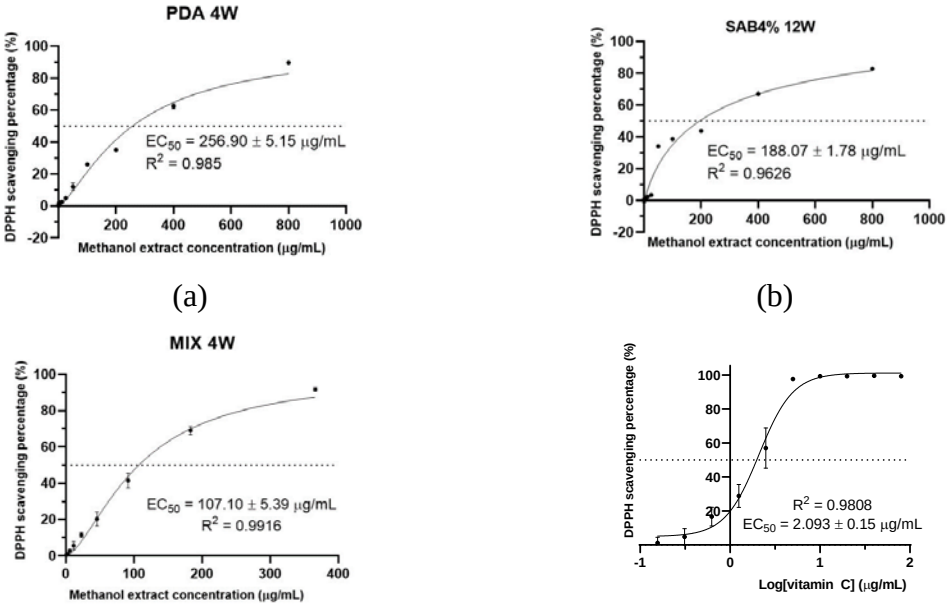


***Figure 3.*** *The relationship between DPPH scavenging percentage and methanolic extract concentrations*

(a) PDA 4W, (b) SAB4% 12W, (c) Mix 4W, (d) Vitamin C

As shown in Figure 3, the correlation curves between the DPPH scavenging percentage and the concentration of methanolic extracts from mycobiont cultured in PDA medium for 4 weeks, SAB4% medium for 12 weeks, and MIX medium for 4 weeks were constructed with high $R^2$ values of 0.985, 0.9626, and 0.9916, respectively. Based on these curves, the

$EC_{50}$ values of the methanolic extracts from mycobiont cultured in PDA medium for 4 weeks, SAB4% medium for 12 weeks, and MIX medium for 4 weeks were determined to be 256.9 ± 5.1, 188.07 ± 1.78, and 107.1 ± 5.39 µg/mL, respectively. Compared to vitamin C ($EC_{50}$ = 2.093 ± 0.15 µg/mL), all three methanol extract samples exhibited higher $EC_{50}$ values, indicating lower DPPH radical scavenging activity.

In summary, the culture medium and culture time affected the DPPH radical scavenging activity of methanolic extracts from *G. handelii* mycobiont.

### *3.3. α – Glucosidase inhibition*

Table 3 presents the specific results of the α-glucosidase enzyme inhibitory activity assay of the nine methanol extracts obtained from the *G. handelii* mycobiont. Overall, eight extracts exhibited weak enzyme inhibitory activity. Among them, the methanol extract derived from the mycobiont cultured on PDA medium for 12 weeks displayed the highest inhibition (6.43%). Conversely, the methanol extract derived from the mycobiont cultured on SAB4% medium for 12 weeks showed no inhibitory activity.

***Table 3.*** *Percentage of α-glucosidase inhibition of extracts at the concentration of 200 µg/mL*

| No | Medium | Time of culture (weeks) | | |
|---|---|---|---|---|
| | | 4 | 8 | 12 |
| 1 | PDA | 4.77 ± 0.53 | 3.56 ± 0.42 | 6.43 ± 1.20 |
| 2 | SAB4% | 5.00 ± 1.43 | 3.41 ± 1.62 | 0.01 ± 0.00 |
| 3 | MIX | 3.02 ± 1.14 | 4.01 ± 1.27 | 4.77 ± 1.26 |

As shown in Table 3, the methanol extracts of the *G. handelii* mycobiont cultured on PDA, SAB4%, and MIX media exhibited feeble α-glucosidase inhibitory activity. These findings diverge from those reported by Tran et al. (2024), who observed a relatively high α-glucosidase inhibitory activity with an $IC_{50}$ value (µg/mL) of 50.0 ± 0.6 for the crude extract of the *G. handelii* mycobiont (Tran et al., 2024). Several factors might explain these contrasting results. Firstly, the lichen samples originated from different locations: Lam Dong Province in the previous study and Long An Province in the current study. Variations in geographical location and climate may be one factor that influence lichens' biological activities. Secondly, the culture media differed between the studies. The mycobiont was cultured on PDA, SAB4%, and MIX media, whereas Tran et al. (2024) employed the MY10 medium. Thirdly, the extraction solvents used were distinct. This study used methanol, while the previous used ethyl acetate to obtain the crude extract. The variations in the methodology employed may have influenced the capacity of the *G. handelii* mycobiont to synthesise secondary metabolites.

## 4. Conclusion

This study investigated the effect of culture media and culture time on methanolic extracts' antioxidant and α-glucosidase inhibitory activities from the *G. handelii* mycobiont.

The results revealed that both factors significantly influenced the DPPH radical scavenging activity of the extracts. Extracts obtained from MIX medium after four weeks of culture exhibited the strongest activity (86.52%), with the $EC_{50}$ of 107.1 ± 5.39 µg/mL. Conversely, the effect of culture medium and time on α-glucosidase inhibitory activity was less pronounced. All extracts exhibited weak or no inhibitory activity, with the highest observed in the extract from the PDA medium after 12 weeks (only 6.43 ± 1.20 %). These findings highlight the crucial role of culture conditions in optimising the biological activity of *G. handelii* mycobiont extracts. While this study revealed the influence of culture conditions on the antioxidant activity of *G. handelii* mycobiont extracts, it did not identify the optimal medium and culture time for strong alpha-glucosidase inhibition. Therefore, further research is necessary to optimise culture conditions for maximising alpha-glucosidase inhibitory activity.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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# ẢNH HƯỞNG CỦA MÔI TRƯỜNG VÀ THỜI GIAN NUÔI CẤY ĐẾN HOẠT TÍNH CHỐNG OXY HÓA VÀ ỨC CHẾ A-GLUCOSIDASE CỦA CAO METHANOL TỪ NẤM CỘNG SINH ĐỊA Y *Graphis handelii*

***Nguyễn Thành Đạt[1], Nguyễn Hồng Linh[1], Phạm Trương Phú Vạn Tài[1], Võ Thị Phi Giao[2], Trần Thị Minh Định[1*]***

*[1]Trường Đại học Sư phạm Thành phố Hồ Chí Minh, Việt Nam*

*[2]Trường Đại học Khoa học Tự nhiên, Đại học Quốc gia Thành phố Hồ Chí Minh, Việt Nam*

*[*]Tác giả liên hệ: Trần Thị Minh Định – Email: dinhttm@hcmue.edu.vn*

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## TÓM TẮT

*Nghiên cứu này đánh giá ảnh hưởng của môi trường và thời gian nuôi cấy đến hoạt tính chống oxy hóa và ức chế alpha-glucosidase của cao methanol thu được từ nấm cộng sinh địa y Graphis handelii. Ba môi trường nuôi cấy khác nhau gồm MIX, PDA và SAB4% được sử dụng để nuôi cấy nấm, và sinh khối được thu hoạch ở các thời điểm nuôi cấy khác nhau (4, 8 và 12 tuần). Các cao methanol thu được sau đó được đánh giá khả năng bắt gốc tự do và ức chế enzyme alpha-glucosidase. Kết quả cho thấy tất cả chín cao methanol đều có khả năng loại gốc DPPH in vitro. Trong số đó, cao methanol từ nấm cộng sinh nuôi cấy trong môi trường MIX trong 4 tuần có hoạt tính mạnh nhất, loại được 86,52% DPPH ở nồng độ 200 µg/mL và giá trị $EC_{50}$ được xác định là 107,1 ± 5,39 µg/mL. Ngược lại, tất cả các cao đều có hoạt tính ức chế enzyme α-glucosidase yếu hoặc không ức chế α-glucosidase. Phát hiện này cho thấy thành phần môi trường nuôi cấy ảnh hưởng đến quá trình sản xuất các hợp chất chống oxy hóa của nấm cộng sinh địa y. Nghiên cứu này có ý nghĩa trong việc tối ưu hóa điều kiện nuôi cấy để khai thác tối đa tiềm năng sinh học của cao methanol từ nấm cộng sinh địa y G. handelii, đặc biệt là đối với các hoạt chất chống oxy hóa.*

***Từ khóa:*** *Graphis handelii*; kháng oxi hóa; nấm cộng sinh địa y; ức chế alpha-glucosidase