INVESTIGATION OF BIOLOGICAL ACTIVITIES OF MIXED EXTRACTS OF MEDICINAL MUSHROOMS (*PLEUROTUS CITRINOPILEATUS* AND *CORDYCEPS MILITARIS*) IN PROTECTING HUMAN HEALTH

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ABSTRACT

Medicinal mushrooms have been proposed as a novel therapy that can improve cancer treatment and patient survival. One of the active ingredients that contributes to a strong immune stimulus found abundantly in mushrooms is β-1,3-glucan. This study focuses on the extraction of β-glucan from two medicinal mushrooms, *Pleurotus citrinopileatus* and *Cordyceps militaris*, by the assisted extraction of a combination of ultrasonic waves and pyrolysis in an aqueous solvent at 100°C, then evaluation of the biological activity of the extract to orient the application of functional foods to support cancer treatment. β-glucan was successfully obtained from mushroom powder by the research team. Through some methods to test the biological activity, the research team demonstrated the antioxidant and cell proliferation potential cells of two medicinal mushrooms, *Pleurotus citrinopileatus* and *Cordyceps militaris*. The results obtained in this study showed that the extract containing the combination of these two medicinal mushrooms exhibited outstanding biological activities.

KHẢO SÁT HOẠT TÍNH SINH HỌC CỦA DỊCH CHIẾT HỖN HỢP NẤM DƯỢC LIỆU (*PLEUROTUS CITRINOPILEATUS* VÀ *CORDYCEPS MILITARIS*) TRONG BẢO VỆ SỨC KHỎE CON NGƯỜI

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TỪ KHÓA

Nấm dược liệu
Pleurotus citrinopileatus
Cordyceps militaris
Hoạt tính sinh học
β-glucan

TÓM TẮT

Nấm dược liệu đã được đề xuất như một liệu pháp mới có thể cải thiện việc điều trị ung thư và khả năng sống sót của bệnh nhân. Một trong những hoạt chất góp phần kích thích miễn dịch mạnh được tìm thấy nhiều trong nấm là β-1,3-glucan. Nghiên cứu này tập trung vào quá trình tách chiết β-glucan từ hai loại nấm dược liệu *Pleurotus citrinopileatus* và *Cordyceps militaris* bằng phương pháp chiết xuất nhiệt phân có sự hỗ trợ của sóng siêu âm trong dung môi nước ở nhiệt độ 100°C, sau đó đánh giá hoạt tính sinh học của dịch chiết để định hướng trong ứng dụng bào chế thực phẩm chức năng hỗ trợ điều trị ung thư. Từ bột nấm, nhóm nghiên cứu đã phân lập thành công β-glucan. Thông qua các phép thử nghiệm hoạt tính sinh học nhóm nghiên cứu chứng minh được khả năng chống oxy hóa và tăng sinh tế bào của hai loại nấm dược liệu *Pleurotus citrinopileatus* và *Cordyceps militaris*, đặc biệt kết quả thu được trong nghiên cứu này cho thấy dịch chiết xuất có sự kết hợp giữa hai loại nấm dược liệu trên thể hiện các hoạt tính sinh học vượt trội.

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1. Introduction

Medicinal mushrooms have been proposed as a novel therapy that can improve cancer treatment and patient survival. Many studies have shown that *Cordyceps Sinensis* extract has antioxidant, anti-aging, neuroprotective, nootropic, immunomodulatory, anti-cancer, and hepatoprotective roles [1]. Besides the above mushrooms, *Pleurotus citrinopileatus* is also known as a valuable medicinal herb. *Pleurotus citrinopileatus* extract has been studied for its anti-hyperglycemic, antioxidant, and lipid-lowering properties [2]. One of the active ingredients that contributes to a strong immune stimulus found abundantly in mushrooms is β-1,3-glucan [3]. Currently, techniques for extracting organic compounds from plant materials range from conventional to advanced, non-traditional methods. Several publications have pointed out some advantages of hot water extraction, such as providing the highest yield and purity and demonstrating maximum removal of impurities [4]. However, traditional methods for extracting bioactive compounds from mushroom raw materials have limitations, such as consuming too much time, energy, and extraction solvent. Therefore, new methods, such as ultrasonic-assisted extraction (UAE), are more commonly used, which improves extraction efficiency and reduces extraction time [5].

Hot water extract of *Pleurotus citrinopileatus* scavenges 50% of the initial DPPH radicals (IC_{50}) at concentrations of 4.2 mg/ml [6]. *Cordyceps militaris* also exhibited superior antioxidant activity, with the IC_{50} value of the metholic mushroom extract being 0.72 mg/ml [7]. The aqueous extract of *Pycnoporus sanguineus* exhibited moderate lipid peroxidation inhibitory activity with an IC_{50} value of 1311.24 µg/mL [8]. The IC_{50} value of *Morchella esculenta* extract to inhibit lipid peroxidation was reported by B. Nitha et al. as 420 ± 26.45 µg/ml [9]. Considering the importance of medicinal mushrooms to human health, this study aimed to study the extraction process of two medicinal mushrooms, *Pleurotus citrinopileatus* and *Cordyceps militaris*. This study focuses on the extraction of two medicinal mushrooms, *Pleurotus citrinopileatus* and *Cordyceps militaris*, by the assisted extraction of a combination of ultrasonic waves and pyrolysis in an aqueous solvent at 100°C, then evaluation of the biological activity of the extract to orient the application of functional foods to support cancer treatment.

2. Materials-Methods

2.1. Materials - Equipment

- Materials: Dried *Pleurotus citrinopileatus* powder, dried *Cordyceps militaris* (Mushrooms raised at Liangshan Mushroom Farm by VIETRAP Company), distilled water, 20% sodium carbonate (Na_2CO_3) solution (China), 2M hydrochloric acid (HCl) (China), 99% isopropyl alcohol (IPA) solution (China) and sodium chloride (NaCl).

- Tools and equipments: UV-Vis Spectrophotometer (UV-Vis Drawell D8200), ultrasound machine; heating magnetic stirrer; centrifuge; drying cabinets; refrigerator; pH meter; analytical scales.

2.2. Methods

2.2.1. Extraction process of β-glucan from mushroom powder [10]

- Prepare 3 samples of mushroom powder: S_1: 8.35 g of *Pleurotus citrinopileatus powder*; S_2: 8.35 g *Cordyceps militaris* mushroom powder; S_3: 8.35 g powder of *Pleurotus citrinopileatus* and *Cordyceps militaris* (1:1 g/g)

- Mushroom powder samples were put into a 500 mL beaker, then 2.5 g sodium chloride and 250 mL distilled water were added to the beaker. The mixture was ultrasonic for 60 mins at room temperature. Then, the mixture was stirred at 100°C for 2 hours at a stirring speed of 3/4. The mixture obtained was centrifuged at 3.500 rpm for 15 minutes. After centrifugation, collect the solution after separating the solid (fungal extract).

- The mushroom extract was adjusted to pH = 10 with 20% Na_2CO_3 solution, the solution was magnetically stirred at 50°C for 30 minutes at a speed of 1/4. Then, centrifuge the mixture for 15 minutes at 3,500 rpm. After the second centrifugation, the extract was adjusted to pH = 4 with 2M HCl solution. Add an amount of isopropyl alcohol (IPA) equal to the correct volume of the mixture. The mixture was stored at 4°C for 24 hours and then centrifuged at 3,500 rpm for 15 minutes. The solid was dried at 80°C for 4-6 hours and re-weighed to evaluate performance. Determination of β-glucan by UV-Vis photometric method at 265 nm on a Drawel machine at the Institute of Chemistry-Materials. The procedure for extracting β-glucan from mushrooms is shown in Figure 1.

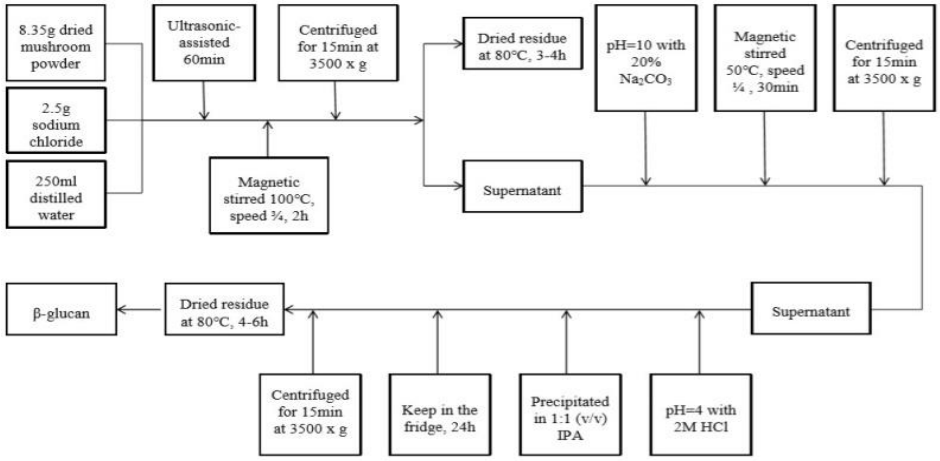

Figure 1. *Extraction process of β-glucan from mushroom powder*

2.2.2. Evaluation of the biological activity of the extracts of medicinal mushrooms Pleurotus citrinopileatus and Cordyceps militaris

DPPH free radical scavenging assay

Each mushroom extract's free radical scavenging capacity was determined according to the method of Abramovič. DPPH was mixed in methanol at a concentration of 0.25 µM. A test sample consisting of 20 µL of the mushroom extract was diluted in 80 µL of methanol, which was then reconstituted into the sample test concentration range with deionized distilled water. Each mixture contained 100 µL of 0.25 µM DPPH solution and 100 µL of the study sample. The mixture was incubated at room temperature in complete darkness for 30 minutes. The absorbance of each sample was measured at 517 nm. The DPPH free radical scanning operation is calculated using the equation:

$$\% SA = (OD_{control} - OD_{sample})*100/OD_{control} \ (\%) \quad (1)$$

Where: $OD_{control}$: Optical density at the well does not contain reagents (minus OD blank)

OD_{sample}: Optical density at the well containing the reagent (minus OD blank)

Inhibition of lipid peroxidation assay (MDA test)

The TBA reaction method estimated lipid peroxidation induced by the Fe2+-ascorbate system in rat liver homogenate. The sample solution was reconstituted into concentrations of 10,000 µg/mL, 2,000 µg/mL, 400 µg/mL, and 80 µg/mL, reacted with 1 mL of liver homogenate and 0.8 mL of phosphate buffer, along with 0.1 mL of phosphate buffer. If the Fenton system is just 2 mL, the sample concentration in the test tube is reduced 20 times to 500 µg/mL, 100 µg/mL, 20 µg/mL, and 4 µg/mL. The reaction mixture was incubated for 15 minutes at 37°C. Stop the reaction with 1 mL of 10% trichloroacetic acid - centrifuge at 12,000 rpm for 5 minutes. Take the clear solution and react it with 1 mL of 0.8% thiobarbituric acid (in a ratio of 2:1). Incubate at 100°C for 15 minutes. Cool and measure at λ = 532 nm. The formula for calculating antioxidant activity percentage (HTCO):

$$HTCO\ (\%) = [(OD_C - OD_T)/OD_C] \times 100 \quad (2)$$

Where: OD_C: Optical density of certification wells without test specimens (minus OD blank)

OD_T: Optical density of test specimen (minus OD blank)

Proliferate cell ability evaluation assay (MTT test)

The MTT assay was used to evaluate the protective and cytotoxic effects of the extract. After adjusting for suitable cell density, insert 190 mL of cells into the wells of a 96-well plate containing 10 µL of reagent. Place the culture plate in the CO_2 incubator at 37°C, 5% CO_2, and culture for 72 hours. After 72 hours, 10 µL of MTT (final concentration of 5 mg/mL) was added to each well. After 4 hours of incubation, the medium was removed 50 µL (DMSO) with 100% Absorbance measured by BioTek spectrophotometer at 540 nm. The percentage of cell proliferation in the presence of a reagent will be determined through the following formula:

$$\%\ proliferation = (OD_{sample} - OD_{blank})/(OD_{DMSO} - OD_{blank}) \quad (3)$$

Where: OD_{sample}: Optical density of the sample

OD_{DMSO}: Optical density of DMSO 1%

OD_{blank}: Optical density of blank well

3. Results and discussion

3.1. Evaluation of β-glucan extraction efficiency in Pleurotus citrinopileatus and Cordyceps militaris mushroom

Measuring UV-Vis spectra from solid samples obtained in experimental samples S_1, S_2, and S_3 the results of maximum UV absorption at wavelengths are 256 nm, 270 nm, and 284 nm, respectively (figure 2).

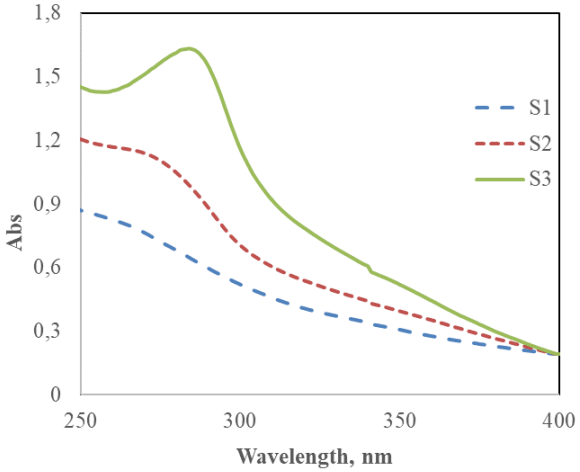

Figure 2. *UV-Vis spectra of β-glucan obtained experimentally of samples S_1, S_2, and S_3*

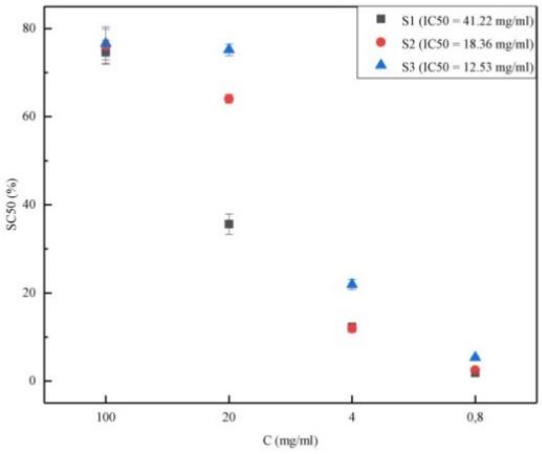

Figure 3. *The DPPH free radical neutralization ability of the mushroom extracts S_1, S_2, and S_3*

Compared with previously published results on UV-vis spectra of glucan, the results obtained by the team are similar to those obtained on β-glucan spectroscopy published by Pawadee Methacanon et al. [11] and Shazia Anwer Bukhari et al. [12]. Although it is observed that the absorption peak is different among the studied samples, this result is still consistent with many previous works. Xu et al. reported that the increase in absorbance with decreasing molecular weight (increasing radiation dos) could be due to the formation of a carbonyl group or a double bond of the glucan formed [13]. Thus, the research team has successfully isolated β-glucan from samples of the fungus *Pleurotus citrinopileatus*, *Cordyceps militaris*, and the mixture of *Pleurotus citrinopileatus* and *Cordyceps militaris*.

In addition, figure 2 also shows that the β-glucan content extracted from the mushroom samples was different. S_3, with a mixture of *Pleurotus citrinopileatus* and *Cordyceps militaris* fungi showed superior β-glucan content compared to S_2 (containing only *Cordyceps militaris*) and S_1 (containing only *Pleurotus citrinopileatus*). Because β-glucan is one of the most potent immunostimulant-contributing active substances found in mushrooms, the β-glucan content greatly affects the biological activity of the extracts of S_1, S_2, and S_3 mushroom extracts. The efficiency of β-glucan extraction from S_1, S_2, and S_3 mushroom samples was about 10.13%, 14.45%, and 19.73%, respectively. Compared with previously published results, the yield of β-glucan extracted from experimental samples in this study was higher than that of β-glucan extracted from wheat in the Lena et al. efficiency value study is 7.1% [14]. However, with the yield values obtained, the yield of β-glucan in each mushroom was smaller than that of barley malt (14.8%) and rye (20.3%) [14].

3.2. Evaluation of the biological activity of the extracts of medicinal mushrooms Pleurotus citrinopileatus and Cordyceps militaris

3.2.1. DPPH free radical scavenging assay

As shown in Figure 3, the DPPH radical scavenging capacity of the samples increased sharply with increasing concentration. With IC_{50} value = 18.36 ± 1.03 (mg/ml), *Cordyceps militaris* demonstrated higher DPPH free radical neutralization ability than *Pleurotus citrinopileatus* with IC_{50} value = 41.22 ± 2.94 (mg/ml). When performing the combination of two fungi, *Pleurotus citrinopileatus* and *Cordyceps militaris* (1:1 g/g), the obtained extract showed better antioxidant capacity through DPPH free radical scavenging test with IC_{50} = 12.53 ± 1.27 (mg/ml). With the obtained IC_{50} values, two medicinal mushrooms, *Pleurotus citrinopileatus*, and *Cordyceps militaris*, have potential applications as functional foods to support cancer treatment, opening up new research directions such as combining both mushrooms to optimize antioxidant capacity. However, the mushroom samples' DPPH free radical neutralization capacity in this study was lower than previously published results. It is possible that the ultrasonic processing is not optimal, leading to the low ability of DDPH free radical scavenging of the extracted extract. To improve this biological activity of the extract, further research is needed to apply the product to the preparation of functional foods to support cancer treatment.

3.2.2. Inhibition of lipid peroxidation assay (MDA test)

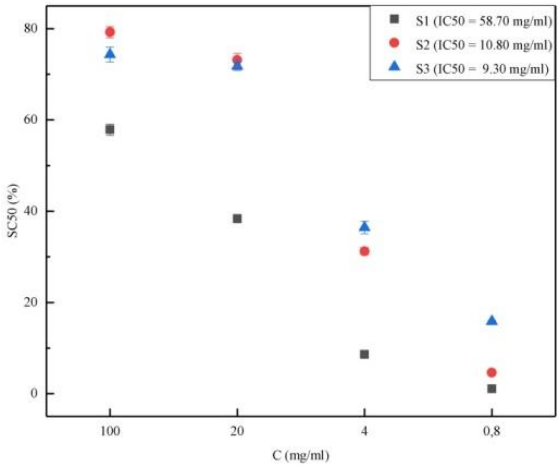

Figure 4. *Ability to inhibit lipid peroxidation (MDA test) of mushroom extracts S_1, S_2, and S_3*

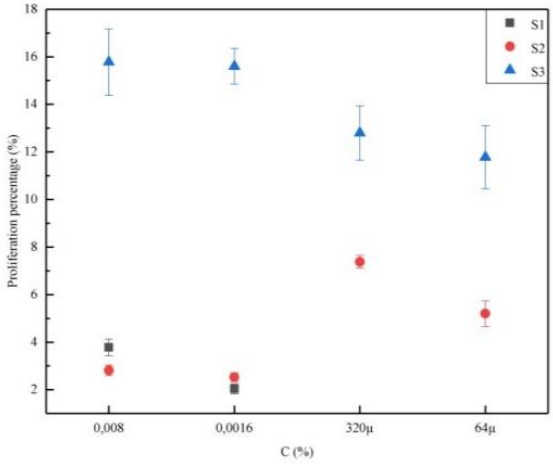

Figure 5. *Macrophage cell proliferation capacity of mushroom extracts S_1, S_2 and S_3*

As shown in Figure 4, the inhibition of lipid peroxidation of the mushroom extracts S_1, S_2, and S_3 enhanced with increasing concentration. Like DPPH free radical scavenger activity, *Cordyceps militaris* extract with IC_{50} = 10.80 ± 0.40 (mg/ml) showed much stronger inhibition of lipid peroxidation than *Pleurotus citrinopileatus* extract with IC_{50} value = 58.70 ± 3.80 (mg/ml), and when combining both medicinal mushrooms, the obtained extract showed a stronger ability to inhibit lipid peroxidation with IC_{50} value = 9.30 ± 0.50 (mg/ml). With the obtained IC_{50} values, two medicinal mushrooms, *Pleurotus citrinopileatus* and *Cordyceps militaris,* have potential applications as functional foods to support cancer treatment, opening up new research directions, such as combining both mushrooms to optimize the ability to inhibit lipid peroxidation. However, the inhibitory ability of the mushroom extracts in this study was very weak compared with *Morchella esculenta* mushroom extract (IC_{50} = 420 ± 26.45 µg/ml) [9] and water extract-ethanol of *Inonotus obliquus* (IC_{50} = 1.23 mg/ml) [15]. Therefore, to improve this biological activity of the extract, further research is needed to apply the product to prepare functional foods to support cancer treatment.

3.2.3. Proliferate cell ability evaluation assay (MTT test)

The research team evaluated the macrophage cell proliferation ability of the mushroom extracts in this study. The results in Figure 5 show that cell viability and growth are affected by the concentration of the sample solution, and the experimental sample S_3, with the combination of two fungi, *Pleurotus citrinopileatus* and *Cordyceps militaris,* has shown the ability to proliferate its superior macrophage cells compared with the fungal samples S_1 and S_2. *Pleurotus citrinopileatus* mushroom extract had the weakest ability to proliferate macrophages when it only showed proliferation-inducing activity at a concentration of 0.0016%. *Cordyceps militaris* mushroom extract showed better activity in inducing macrophage cell proliferation. When showing this activity at a concentration of $6.4x10^{-5}$ %, the cell proliferation ability of *Cordyceps militaris* mushroom extract showed the best activity at a concentration of $3.2x10^{-4}$ %, then the percentage of cell proliferation decreased significantly with increasing concentration. The remarkable point here is that when extracting the mixture of 2 samples of *Pleurotus citrinopileatus* and *Cordyceps militaris*, the obtained extract showed the ability to increase macrophage cells effectively and was much superior to the extract of the samples' separate mushrooms. Our results provide experimental evidence that the aqueous extract of a mixture of the fungi *Pleurotus citrinopileatus* and *Cordyceps militaris* is a potential source of antioxidant and antitumor compounds.

4. Conclusion

Through the research process, referring to the previously published β-glucan extraction process, the medical mushrooms extraction process was determined through the study as follows: The solvent used for extraction was water, the sample was ultrasonically treated for 60 minutes, and the magnetic strip at 100°C for 2 hours, the concentration of Na_2CO_3 participating in the reaction is 20%, the concentration of HCl is 2M, the ratio of treated IPA/fungal residue is 1:1 (v/v). From the *Pleurotus citrinopileatus* and *Cordyceps militaris* mushroom powder, β-glucan was successfully obtained. With the results obtained in the bioactivity assays, the extracting of β-glucan from both *Pleurotus citrinopileatus* and *Cordyceps militaris* fungi have good antioxidant capacity and are capable of cell proliferation macrophages. However, with IC_{50} values of 12.53 ± 1.27 (mg/ml) and 9.30 ± 0.50 (mg/ml), respectively, in DPPH free radical neutralization assays, inhibition of lipid peroxidation assay, combined extracts of two fungi *Pleurotus citrinopileatus* and *Cordyceps militaris* showed superior antioxidant capacity, moreover, this extract also demonstrated the ability to proliferate macrophage cells in the proliferate cell ability evaluation assay.

REFERENCES

[1] P. Giridhar, K. U. Sankar, B. Manohar, and M. G. Shashidhar, "Bioactive principles from Cordyceps sinensis: A potent food supplement–A review," *Journal of Functional Foods*, vol. 5, no. 3, pp. 1013-1030, 2013.

[2] M. M. Abdel-Aziz, M. Yosri, and A. M. Younis, "Evaluation of some biological applications of Pleurotus citrinopileatus and Boletus edulis fruiting bodies," *Current pharmaceutical biotechnology*, vol. 20, no. 25, pp. 1309-1320, 2019.

[3] P. Michaud and C. Laroche, "New developments and prospective applications for β (1, 3) glucans," *Recent patents on biotechnology*, vol. 1, no. 1, pp. 59-73, 2007.

[4] S. Sowrirajan, B. Joseph, and G. Maheshwari, "Extraction and isolation of β-glucan from grain sources—A review," *Journal of Food Science*, vol. 82, no. 7, pp. 1535-1545, 2017.

[5] N. Rombaut, A. G. Sicaire, A. Meullemiestre, A. S. Fabiano-Tixier, M. Abert-Vian, and F. Chemat, "Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols, and applications. A review," *Ultrasonics sonochemistry*, vol. 34, pp. 540-560, 2017.

[6] S. Furuta, S. Fukamizu, R. Yamamoto, H. Ishikawa, E. T. Arung, K. Shimizu, S. Ohga, R. Kondo, and T. X. Meng, "Evaluation of biological activities of extracts from the fruiting body of Pleurotus citrinopileatus for skin cosmetics," *Journal of wood science*, vol. 57, pp. 452-458, 2011.

[7] A. Sagar, S. S. Kanwar, S. Singh, and M. Joshi, "Anticancer, antibacterial and antioxidant activities of *Cordyceps militaris*," *Indian Journal of Experimental Biology*, vol. 57, pp. 15-20, 2019.

[8] D. X. Chu, B. T. Dieu, and T. D. Tuong, " Antioxidant activity of fruiting body extracts from Pycnoporus sanguineus mushroom," *Vietnam Journal of Science and Technology*, vol. 58, no. 2, pp. 143-151, 2020.

[9] S. De, S. K. Adhikari, T. P. Devasagayam, K. K. Janardhanan, and B. Nitha, "Evaluation of free radical scavenging activity of morel mushroom, Morchella esculenta mycelia: a potential source of therapeutically useful antioxidants," *Pharmaceutical Biology*, vol. 48, no. 4, pp. 453-460, 2010.

[10] T. T. X. Nguyen, T. P. A. Nguyen, M. A. Le, H. A. Nguyen, M. H. Nguyen, and T. H. P. Nguyen, "Study of the extraction process of the *Pleurotus citrinopileatus* mushroom and evaluation of the biological activity of the extract," *International Journal of Advanced Engineering Research and Science*, vol. 10, no. 4, pp. 120-127, 2023.

[11] U. Weerawatsophon, P. Tanjak, P. Rachtawee, W. Prathumpai, and P. Methacanon, " Interleukin-8 stimulating activity of low molecular weight β-glucan depolymerized by γ-irradiation," *Carbohydrate polymers*, vol. 86, no. 2, pp. 574-580, 2011.

[12] F. Anjum, M. Shahid, A. Parveen, N. Rasool, M. Zia-Ul-Haq, H. Doğan, S. Ercişli, and S. Bukhari, "Analytical characterisation of beta-glucan isolated from fruiting body of pleurotus OSTREATUS JACQ. EX FR.," *Proceedings of the Bulgarian Academy of Sciences*, vol. 67, no. 5, pp. 655-664, 2014.

[13] Y. Sun, Y. Yang, J. Ding, J. Pang, and Z. Xu, "Effect of γ-irradiation on some physiochemical properties of konjac glucomannan. Carbohydrate Polymers," *Carbohydrate Polymers*, vol. 70, no. 4, pp. 444-450, 2007.

[14] T. Stenberg, R. Andersson, A. Andersson, P. Åman, and L. Rimsten, "Determination of β-glucan molecular weight using SEC with calcofluor detection in cereal extracts," *Cereal Chemistry*, vol. 80, no. 4, pp. 485-490, 2003.

[15] Y. Wu, H. Chen, and X. Xu, "Comparative antioxidative characteristics of polysaccharide-enriched extracts from natural sclerotia and cultured mycelia in submerged fermentation of Inonotus obliquus," *Food Chemistry*, vol. 127, no. 1, pp. 74-79, 2011.