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Study of the extraction process of the Pleurotus citrinopileatus mushroom and evaluation of the biological activity of the extract

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Keywords*— *β-glucan, Pleurotus citrinopileatus, biological activity, functional food.

***Abstract*—** *Pleurotus citrinopileatus, also known in Vietnam as golden oyster mushroom, is a food rich in protein, fiber, essential amino acids, carbohydrates, water-soluble vitamins, and minerals. In particular, in the composition of yellow oyster mushrooms, there is β -1.3 - glucan with high biological activity as an active ingredient contributing to the intense stimulation of immunity. The study researched the process of extracting Pleurotus citrinopileatus mushroom 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. Some of the conditions of the Pleurotus citrinopileatus extraction process documented through the study are as follows: The solvent used for extraction was water, sodium chloride salt was used as a catalyst, with a ratio of 1g NaCl:100 ml of solution, the sample was ultrasonically processed for 60 min and magnetic stirred at 100°C for 2 hours, the concentration of Na_2CO_3 participating in the reaction was 20%, HCl concentration was 2M, the ratio of IPA treatment/mushroom residue was 1:1 (v/v). From Pleurotus citrinopileatus mushroom powder, the research team successfully obtained β- glucan.*

I. INTRODUCTION

Functional food was first used by the Japanese in the 1980s to refer to processed foods that contain ingredients that have little nutritional value but help improve users' health [1]. Pleurotus citrinopileatus is rich in protein, fiber, essential amino acids, carbohydrates, water-soluble vitamins, and minerals [2]. With 22.10% protein and 20.78% fiber while fat only 1.32%, Pleurotus citrinopileatus is a nutritionally balanced food that is good for the body's absorption [3]. Besides, Pleurotus citrinopileatus also has many vitamins and minerals, supplementing trace elements for the body [3]. A study in the journal "Agricultural and Food Chemistry" demonstrated that a glycoprotein in this mushroom, symbol PCP-3A, is very effective in treating blood cancer [4]. Pleurotus citrinopileatus mushroom extract contains a large amount of Ergothioneine, which has a strong antioxidant effect and a substantial effect on the immune system [5].

One of the active ingredients that contributes to a strong immune stimulus found abundantly in Pleurotus citrinopileatus mushrooms is β-1,3-glucan [6]. β-glucan,

when entering the body, will produce two effects: Activating cells of the immune system to ensure they function at the most optimal level; Rapid increase in the number of immune cells [7][8]. β-glucan - a polysaccharide of natural origin present in the cell walls of plants, bacteria, and fungi [9] [10]. Some studies have shown that β-glucan helps improve health status in several ways, such as immunomodulatory, antitumor, antiviral [11] [12], cardiovascular activities, hepatoprotective, anti-inflammatory [13], radiation protection [14], anti-diabetic [15], antioxidant [16], antibacterial [17]. β-Glucan enhances adaptive immune responses that inhibit tumor growth and metastasis [18] [19] and is approved as an immuno-supportive therapy in cancer treatment in some countries [20]. Because of the great uses of β-glucan, β-glucan extraction is always of interest. Many studies have been conducted aimed at obtaining high-purity β-glucan. Some of the main extraction methods used are hot water extraction [16], alkaline extraction [22], enzymatic extraction [23], and ultrasound/microwave-assisted extraction [24]. This paper presents some research results on the process of extracting β-glucan from Pleurotus citrinopileatus by the method of assisted extraction of a combination of ultrasonic techniques 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 to orient the application of functional foods to help cancer treatment.

II. EXPERIMENTS

2.1. Chemicals - Equipment

- Chemicals: Dried Pleurotus citrinopileatus powder (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. Extraction process of β-glucan from Pleurotus citrinopileatus

- Weigh precisely 16.7g of dried Pleurotus citrinopileatus mushroom powder in a 1000ml cup, then add 5g of sodium chloride and 500 ml of distilled water to the cup. Ultrasound was conducted for 60 min at room temperature, after which the mixture was stirred at 100°C for 2 hours at a stirring rate of 3/4. Take the resulting mixture to centrifugation at 3,500 rpm for 15 minutes. After centrifugation, try the solution after separating the solid residue (mushroom extract).

- The mushroom extract was adjusted to pH = 10 with a 20% solution of Na_2CO_3, then stirred at 50°C for 30 minutes at 1/4 speed. Then, centrifuging the mixture for 15 minutes at 3,500 rpm, the extract after the second centrifugation was adjusted to pH = 4 with a 2M HCl solution. Add the correct amount of isopropyl alcohol (IPA) to the volume of the mixture, then continue centrifuging at 3,500 rpm for 15 minutes. The pulp is dried at 80°C for 4-6 hours, weighing the volume is again to evaluate performance. Determination of β-glucan by UV-Vis photometric method at a wavelength of 265 nm on the Drawel instrument at the Institute of Chemistry-Materials.

The content of β-glucan extracted from the extraction technology process is calculated according to the formula:

$$m_{\beta\text{-glucan (theoretical)}} = m_{P.citrinopileatus} \times 21{,}4\% \quad (1)$$

Where: $m_{\beta\text{-glucan (theoretical)}}$: Theoretical β-glucan mass

$m_{P.citrinopileatus}$: Mass of mushroom powder Pleurotus citrinopileatus

21,4%: Percentage of β-glucan mass in Pleurotus citrinopileatus

The formula calculates the extraction efficiency:

$$H = (m_{\beta\text{-glucan (experimental)}}/m_{\beta\text{-glucan (theoretical)}})*100\% \quad (2)$$

Where: H: Extraction efficiency of β-glucan from Pleurotus citrinopileatus

$m_{\beta\text{-glucan (experimental)}}$: Experimantal β-glucan mass

$m_{\beta\text{-glucan (theoretical)}}$: Theoretical β-glucan mass

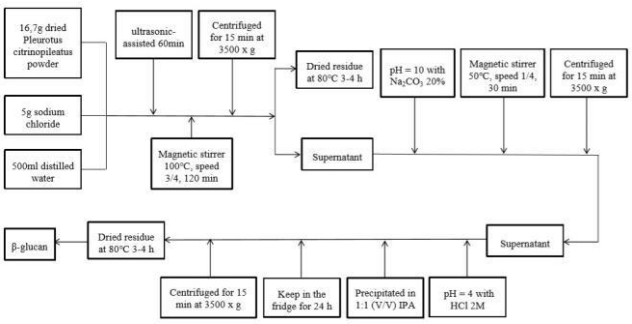

Fig 1. Extraction process of β-glucan from Pleurotus citrinopileatus

2.3. Evaluation of β-glucan extraction from Pleurotus citrinopileatus

Based on UV-Vis photometric analysis technique to evaluate the β-glucan content in samples with different extraction conditions. The β-glucan content calibration in

aqueous solvents is formulated from 100ppm, 200ppm, 300ppm, 400ppm, and 500ppm.

The calibration curve for the β-glucan content of water at a characteristic wavelength (257 nm) is shown in Figure 1. The calibration equation is:

$$y = 95{,}487x + 1{,}335 \quad (3)$$

Where: y: concentration of β-glucan (ppm)

x: adsorption intensity (au)

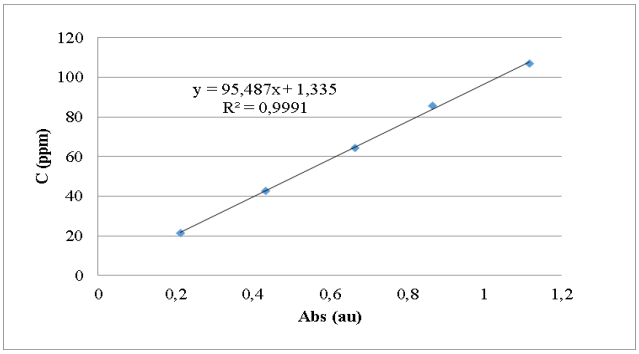

Fig 2.β-glucan content calibration curve

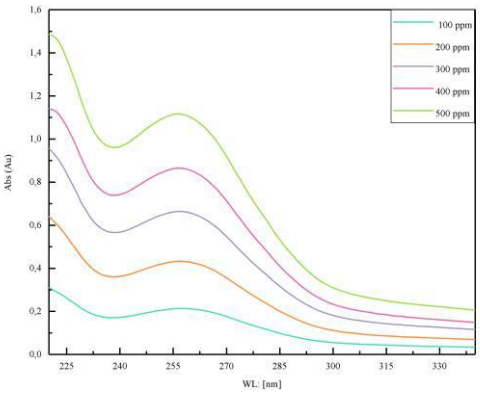

Fig 3. UV-Vis spectrum of β-glucan

2.4 Evaluation of the antioxidant capacity and cell proliferation ability of Pleurotus citrinopileatus mushroom extract

2.4.1 DPPH antioxidant capacity evaluation test

Mushroom extracts were determined for antioxidant capacity using the DPPH method by Abramovič et al. (2018) with modifications to suit laboratory conditions. The suction of the studied sample (100 µL) mixed at concentrations of 100 – 20 – 4 – 0.8 mg/mL, add 100 µ L DPPH 0.25 µM in methanol (100%), then incubate at room temperature for 30 minutes in the dark. The absorption of the reaction is read at a wavelength of 517 nm. The DPPH free radical scanning operation is calculated using the equation:

$$\%\ SA = (OD_{control} - OD_{sample})*100/OD_{control}\ (\%) \quad (4)$$

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)

2.4.2 MDA antioxidant capacity evaluation test

- Mushroom extracts were determined for their antioxidant capacity through MDA testing performed according to the methods of Badmus et al. (2011) and Hodges et al. (1999), with slight modifications to suit laboratory conditions. Aspiration of the studied sample (0.2 mL) mixed at concentrations of 1000 – 200 – 40 – 8 mg/mL reacted with 1 mL of isologous liver fluid and an additional 0.7 mL phosphate buffer, together with 0.1 mL of the Fenton system. The system is sufficient for 2 mL, and the sample concentration in the test tube is reduced ten times to 100 – 20 – 4 – 0.8 mg / mL.

- Take 1mL of mouse liver isotope fluid added to 0.1 mL of the test sample at concentrations and 0.8mL phosphate buffer, and add 0.1mL of Fenton system ($FeSO_4$ 0,1 mM: H_2O_2 15 mM at 1:1 ratio). Incubate the mixture at 37°C for 15 minutes.

- Stop the reaction with 1 mL of 10% trichloroacetic acid. Centrifugal 12000 turns in 5 minutes.

- Clear fluid reacts with 1 mL of 0.8% thiobarbituric acid (in a ratio of 2:1). Incubate at 100°C for 15 minutes. Cool and take measurements at wavelength λ = 532 nm.

The formula for calculating antioxidant activity percentage (HTCO)

$$HTCO\ (\%) = [(ODC - ODT)/ODC] \times 100 \quad (5)$$

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

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

2.4.3 MTT proliferate cell ability evaluation test

- Mushroom extracts were determined for immune cell proliferation through MTT testing by Tim Mosmann (1983) with macrophage cell lines at the Institute of Biotechnology.

- After adjustment for appropriate cell density, put 190 mL of cells into the wells of the 96-well tray that already had 10 µL of reagent. On the same test dish, several wells were arranged as a control with no test samples, only the sample phase solvent was DMSO 1%. The well has no cells and specimens, only the culture medium is considered a blank wall.

- Place the culture dish in the CO_2 incubator at 37°C, 5% CO_2, culture for 72 hours.

- After 72 hours, 10 µL MTT (final concentration is 5 mg/mL) is added to each well.

- After 4h, removing the medium, formazan crystals are dissolved by 50 µL (DMSO) 100%. The OD value was measured at a wavelength of 540 nm using a BioTek spectrometer.

The percentage of cell proliferation induction in the presence of a reagent will be determined through the following formula:

$$\% \text{ proliferation} = (OD_{sample} - OD_{blank})/(OD_{DMSO} - OD_{blank}) \quad (6)$$

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

OD_{DMSO}: Optical density of DMSO 1%

OD_{blank}: Optical density of blank well

III. RESULT AND DISCUSSION

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

Conducting UV-Vis spectroscopy measurements from solid samples obtained a maximum UV absorption result at a wavelength of 256 nm (figure 4), similar to the results obtained on β-glucan spectroscopy published by Pawadee Methacanon et al. (2011). Thus, the team successfully isolated β-glucan from Pleurotus citrinopileatus.

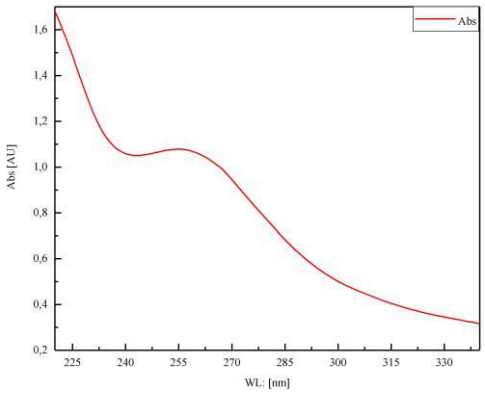

Fig 4. UV-Vis spectroscopy of experimentally obtained β-glucan

From 16.7 grams of Pleurotus citrinopileatus mushroom powder after the extraction process, 0.363 grams of β- glucan is obtained. The efficiency of the β-glucan extraction process in Pleurotus citrinopileatus mushrooms is about 10.157%.

Effects of extraction techniques

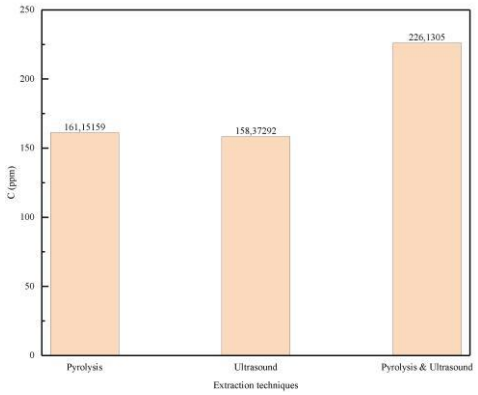

Fig 5.Effect of technique on the β-glucan content obtained

In addition to conventional pyrolysis extraction techniques, the research team surveyed with ultrasound-assisted extraction techniques. The results showed that the extracted β-glucan content was optimal under the same extraction conditions when combining two methods, pyrolysis, and ultrasound (figure 5). This is explained by the cavitation phenomenon caused by ultrasonic waves, this energy destroys the cell wall, allowing water to penetrate higher into which cell wall of the mushroom powder particle. Bhaskaracharya et al. (2009) and Patist and Bates (2008) both believe that the energy generated from bursting air bubbles (cavitation) breaks cell walls and increases the release of cellular materials such as polysaccharides. However, if the ultrasound time is increased, there will be a decrease in the β-glucan extraction yield. This indentation is due to the side effect of ultrasound waves on destroying the β-glucan chain [24]. Therefore, when combining two pyrolysis and ultrasonic techniques, the research team developed a shorter ultrasound time than the pyrolysis time.

Effect of extraction temperature

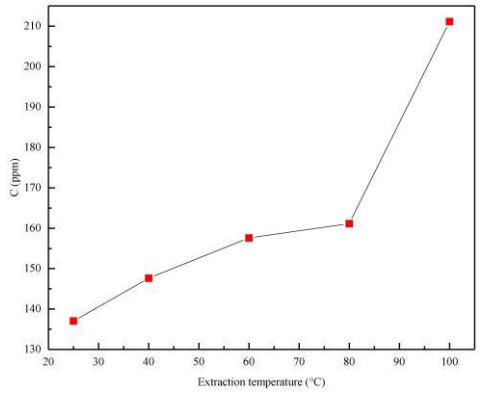

Fig 6.Effect of temperature on the β-glucan content obtained

Figure 6 shows a significant effect of temperature on the extraction yield of β-glucan. The β-glucan content gradually increased from 40°C to 80°C and spiked from 80°C to 100°C. The solvent used is water, with a boiling point of 100°C. Based on the severity coefficient equation to evaluate the hydrolysis reactions [25], the research team surveyed the extracted β-glucan content. at different temperatures (room temperature - 40°C - 60°C - 80°C - 100°C). The obtained β-glucan content increased with temperature. The change in water properties when the temperature is increased to 100°C helps to weaken the bond between the studied polysaccharide and the fungus Pleurotus citrinopileatus, promoting the release of β-glucan. However, Óscar Benito-Román et al. (2016) have shown that if the temperature is raised above 100°C, the hydrolysis kinetics will increase significantly and dominate the increase in solubility [26]. Specifically, maximum β-glucan content is rapid at temperatures above 100°C, hydrolysis of soluble β-glucan occurs almost instantaneously, with major products being detected. In this process are: cellobiose, glucose, fructose, pyruvaldehyde, and HMF [26]. Therefore, the team used 100°C in an aqueous solvent as the optimal β-glucan extraction condition.

Effects of extraction time

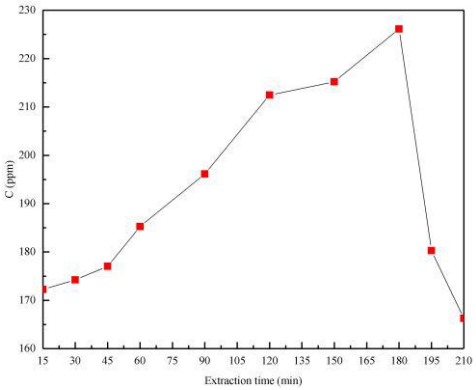

Fig 7.Effect of extraction time on β-glucan content obtained when extracted at 100°C

In addition to temperature, time also affects the performance of the extraction process. The content of extracted β-glucan gradually increases with increasing extraction time from 15 minutes to 3 hours and decreases sharply when the extraction time is more than 3 hours. With the same extraction temperature of 100°C, the results in Figure 7 shows that the longer the β-glucan is exposed to high temperatures, the higher the decomposition, so β-glucan after being dissolved, the sterilization process (rated in MW) takes place, the longer the high-temperature exposure, the lower the MW. Therefore, after investigating the β-glucan content extracted at 100°C for different periods, the research team concluded that the maximum extraction time of β-glucan from Pleurotus citrinopileatus is 3 hours. The content decreases markedly after 3 hours of extraction.

Effect of catalyst

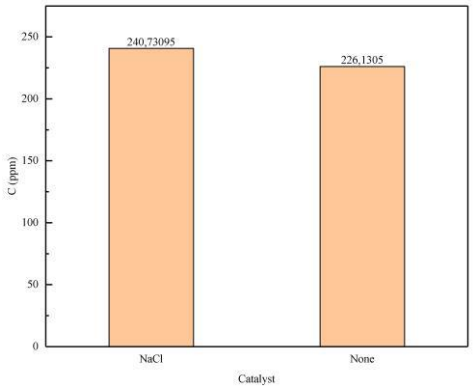

Fig 8. Effect of catalyst on the β-glucan content obtained

After evaluating the effects of extraction technique, temperature, and time on the process, the team investigated the impact of the catalyst on the β-glucan content obtained. Sodium chloride salt is used as a catalyst, with a ratio of 1g catalyst: 100 ml of solution. Figure 8 shows that the β-glucan content obtained is higher when using NaCl as a catalyst. Adding NaCl decreases the solubility of organic analytes and increases the distribution constant, reducing the organic analytes' solubility in the aqueous phase due to the salting-out effect, which is often favorable for extraction [27]. Therefore, the research team proposed the conditions for extracting β-glucan from Pleurotus citrinopileatus: Catalyst ratio 1g NaCl: 100ml solution, combining two pyrolysis techniques and ultrasound-assisted, extraction temperature is 100°C and extraction time is 3 hours.

3.2. Evaluation of the antioxidant capacity of Pleurotus citrinopileatus mushroom extract

3.2.1 Antioxidant Activity Test (DPPH Method)

The results of determining the antioxidant activity of mushroom extract through the DPPH free radical neutralization test are shown in Figure 8. DPPH's ability to neutralize free radicals increases as the concentration of the sample increases. Pleurotus citrinopileatus mushroom extract demonstrated antioxidant activity through DPPH's free radical neutralization test with IC values of 50 = 41.22 ± 2.94 (mg/ml). This indicates that Pleurotus citrinopileatus mushroom extract exhibits antioxidant capacity, so Pleurotus citrinopileatus mushroom can

potentially apply functional foods to support cancer treatment.

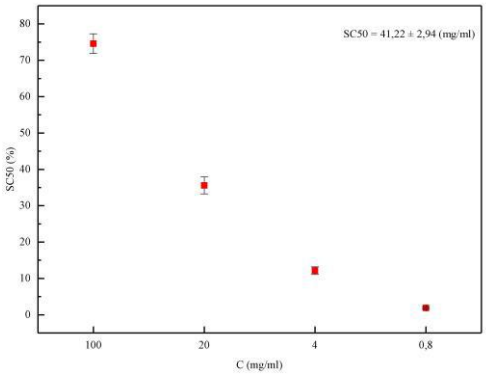

Fig 9. DPPH's ability to neutralize free radicals of mushroom extracts

3.2.2 Lipid Antioxidant Activity Test (MDA Method)

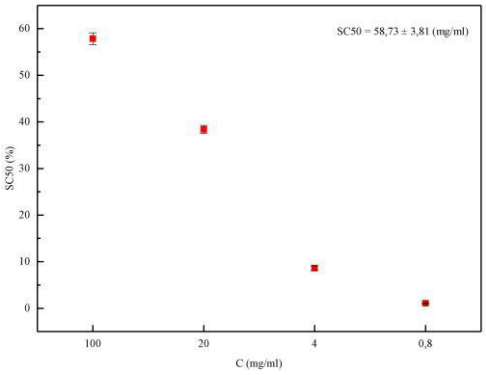

Fig 10.Ability to inhibit lipid peroxidation (MDA test) of mushroom extract

Lipid peroxidation is a mixture of extreme reaction products of lipid peroxidation, a standard process in all biological systems, and hurts cell membranes and DNA [28]. Malondialdehyde (MDA) is a primary oxidation product of peroxidized polyunsaturated fatty acids, and low MDA content has been used as an essential indicator of cell membrane lipid peroxidation [29]. As shown in Figure 9, the inhibition of lipid peroxidation of Pleurotus citrinopileatus mushroom extract intensifies with increasing concentration. The above results show that Pleurotus citrinopileatus mushroom extract exhibited antioxidant activity through lipid peroxidation inhibition test with IC_{50} = 58.73 ± 3.81 (mg/ml). Therefore, Pleurotus citrinopileatus mushroom can potentially apply functional foods to support cancer treatment.

3.3 Evaluation of cell proliferation capacity of Pleurotus citrinopileatus mushroom extract

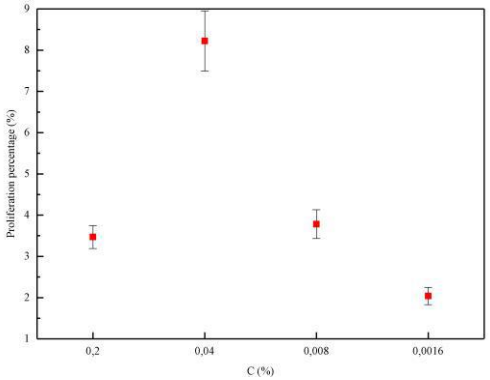

Fig 11.Macrophage cell proliferation capacity of mushroom extract

The cell proliferation capacity of Pleurotus citrinopileatus extract was evaluated through MTT, using tetrazolium salt (MTT-(3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium)) as a reagent in colorimetry, thereby assessing cell survival and growth. The results shown in Figure 10 show that the cell's ability to survive and grow is affected by the sample solution concentration. Pleurotus citrinopileatus mushroom extract exhibits macrophage cell proliferation induction activity at a concentration of 0.04%, the percentage of cell proliferation decreases significantly with increasing concentration. Thus, the extract of Pleurotus citrinopileatus mushroom can promote cell survival and proliferation, opening up potential applications in preparing functional foods to support cancer treatment.

3.4 Evaluation of the stability of Pleurotus citrinopileatus mushroom extract

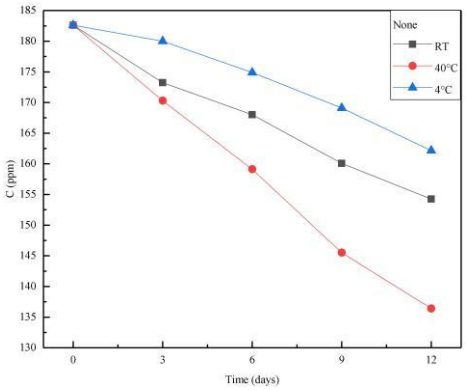

Fig 12. Stability of the extract under the additive-free condition

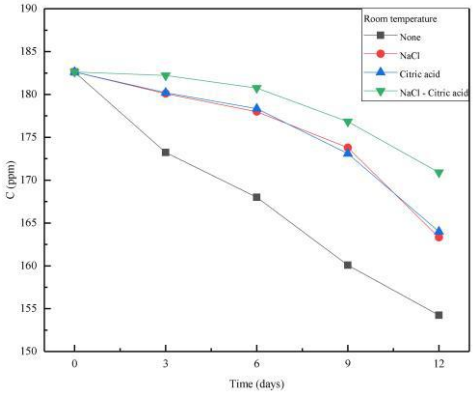

Fig 13. Stability of the extract under the additive condition

The research team evaluated the stability of the Pleurotus citrinopileatus mushroom extract in the laboratory for one week, provided there were no additives (Figure 12) and additives (Figure 13). The results in Figure 12 show that after a week of storage in the absence of additives, the β-glucan content decreased rapidly at 40°C, gradually decreased at room temperature, and was the least changed at 4°C. Besides, the stability of the extract is also affected in the presence of additives. Figure 13 shows that, after one week, the retention of β-glucan was higher in the fact of the additive, and it was highest in the presence of the two additives, NH_4Cl, and citric acid. These results supported our hypothesis that the β-glucan content in the mushroom extract can break down over time and that storage conditions will affect their stability. The ideal conditions for preserving the extract are refrigerated storage and the presence of two additives, NH_4Cl, and citric acid.

IV. CONCLUSION

Through the research process, the research team determined the optimal parameters for the Pleurotus citrinopileatus extraction process recorded through the study as follows: The solvent used for extraction was water, the sample was ultrasonically treated for 60 min and magnetic stirred 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 mushroom powder Pleurotus citrinopileatus, the thematic group successfully obtained β- glucan. Pleurotus citrinopileatus mushroom extract achieved antioxidant capacity through DPPH free radical neutralization test with IC value 50 = 41.22 ± 2.94 (mg/ml) and lipid peroxidation inhibition test with IC value50 = 58.73 ± 3.81 (mg/ml). In addition, mushroom extract can proliferate cells through MTT testing, which proves that Pleurotus citrinopileatus extract is a potential food source in the application of cancer treatment supplements.

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