FROZEN GRANULATION OF ETHANOL EXTRACT OF BROWN OYSTER MUSHROOM (*Pleurotus cystidiosus*): MYCOCHEMICAL PROFILE BASED ON FOURIER TRANSFORM INFRARED (FTIR) AND ANTIBACTERIAL ACTIVITY

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Abstract. Brown oyster mushroom (*Pleurotus cystidiosus*) is a group of edible mushrooms found in many tropical countries. The utilization of *P. cystidiosus* mushrooms is still limited to daily food applications; even though they have much potential, they are developed into antioxidant, anti-inflammatory, or immunomodulatory supplements. In addition, manufacturing preparations from mushrooms to obtain bioactive compounds is still widely done by extraction methods with the principle of maceration. The maceration method takes a long time and wastes solvents, requiring high costs. Based on this, this study was conducted to prepare a frozen granulation of P. cystidiosus using freeze drying. The extraction process was carried out by sonication using a sonicator at a frequency of 50 Hz for 45 minutes for three running cycles, identifying the content of bioactive compounds using Fourier Transform Infrared (FTIR), and testing the activity of *P. cystidiosus* granules as antibacterial against four pathogenic bacterial species namely Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, and Pseudomonas aeruginosa. Using the disc method. The research method was descriptive-analytical, where the results of compound identification were characterized and identified with an FTIR instrument. At the same time, antibacterial testing was carried out using the MIC macro-broth dilution method with a complete randomized design (CRD) with further tests using the Duncan-multiple range test using SPSS software ver. 27.0 and GraphPad Prism Ver 10.3.0. The results showed that the frozen granule of *P. cystidiosus* mushroom contained many diverse compounds and had the potential to be further developed and isolated to *P. cystidiosus* granules, which can also inhibit the growth of four types of pathogenic microorganisms used in antibacterial testing. The development of *P. cystidiosus* granule preparation can be continued by encapsulating it using a suitable coating and can be continued to be carried out in vivo testing.

Keywords: antibacterial activity, brown oyster mushroom, frozen granulation, FTIR

A. Introduction

The use of antibiotics in patients is very common and widely used by the public. Some antibiotics are sold freely in pharmacies, and some require a doctor's prescription. However, patients often take antibiotics without following procedures and doctor's recommendations, and continuous use can cause resistance. Antibiotic resistance can cause the treatment of patients suffering from microorganism infections to be inefficient and costly [1]. The continuous use of synthesized antibiotics can also cause side effects on kidney health. Therefore, it is necessary to explore and develop herbal-based antibiotics with minimal side effects if used long-term. Brown oyster mushroom (*Pleurotus cystidiosus*) is a group of edible mushrooms widely consumed by people in Indonesia. Mushrooms of the Pleurotus genus have many edible species and contain many bioactive compounds, nutrients, and polysaccharide content that nourish the body. In general, edible mushrooms contain around 19-35% protein. At the same time, the fat content is shallow compared to carbohydrates and protein, so mushrooms are often used as an alternative functional food to replace meat for some people, especially vegetarians [2]. The use of mushrooms as health supplements needs to be developed to make products that are more practical, efficient, and appropriate so that they can be utilized by the wider community [3].

The use of mushrooms has been limited to extracts made through an extraction process to isolate and obtain compounds that can provide health benefits. Still, extract products have many disadvantages, one of which is that they are easily contaminated and require complicated storage procedures. This study used a frozen granule product from *P. cystidiosus* extract, which has a drier, granule-shaped, and frozen dosage form. Frozen granules made through the freeze-drying process are relatively more stable, more durable, not easily contaminated, and can minimize the risk of damage to the bioactive compounds they contain. The use and application of frozen granule products from mushrooms is still very rarely done, including for anti-bacterial testing; so far, researchers have more often used extracts because they are considered simpler and easier to apply. In addition, the identification of the content of mushroom bioactive compounds has been mostly done using qualitative or quantitative methods using a UV-VIS spectrophotometer. Still, identification using Fourier Transform Infrared Spectroscopy (FTIR) has not been done much, especially for frozen granule products from freeze drying, so further research needs to be done in this study. The novelty of this study is to test the effectiveness of frozen granules from *P. cystidiosus* extract as antibacterial against four species of pathogenic bacteria *Listeria monocytogenes ATCC 7664*, *Pseudomonas aeruginoas ATCC 9027*, *Escherichia coli ATCC 8739*, and *Staphylococcus aureus ATCC 25923*, and to determine the content of bioactive compounds through the FTIR method.

B. Materials And Method

The tools used in this research include freeze dryer (VIRTIS: BENCHTOP 2K XL), Fourier-transform infrared spectroscopy (FTIR), Vacuum rotary evaporator, vacuum chamber, shaker incubator, hotplate, magnetic stirrer, aluminum foil (klinpak), stirring rod, dark bottle, blender (Philips), measuring cup (pyrex®), goblet (pyrex®), volumetric flask (pyrex®), object glass (pyrex®), mortar and stamper, pH meter, dropper pipette, measuring pipette (pyrex®), analytical balance, test tube (pyrex®), test tube rack, stopwatch, and stationery. The materials used in this study include four isolates of pathogenic bacteria obtained from the culture laboratory of the National Research and Innovation Agency (BRIN), Playen Gunung Kidul, including *Listeria monocytogenes ATCC 7664*, *Pseudomonas aeruginosa ATCC 9027*, *Escherichia coli ATCC 8739*, and *Staphylococcus aureus ATCC 25923*, selective growth media for each bacteria, 70% disinfectant alcohol, 95% alcohol, sterile distilled water, cotton, tissue paper, wrapper, NA (Nutrient Agar) media, TSA (Tryptone Soy Agar) media, TSB (Tryptone Soy Broth) media, NaCl 0.85%, spirtus, H_2O_2, crystal violet, malachite green dye, safranin

solution, iodine solution, immersion oil, distilled water, sterile cotton, aluminum foil, wrap paper, heat-resistant plastic, and filter paper.

This research was conducted in April-August 2024 for in vitro antibacterial stages, preparation of simplisia, making thick extract of *P. cystidiosus* by sonication method, making frozen granule of *P. cystidiosus* extract, identification of compounds with FTIR, and calculation of IC_{50}. This research method is a true experiment with a complete randomized design (RAL), where antibacterial activity testing is measured by the Minimum Inhibition Concentration (MIC) method. The IC_{50} value is calculated for each test group.

1. Extraction of Pleurotus cystidiosus Mushroom

Extraction of *P. cystidiosus* was performed using the sonication method. 500 g dried simplistic of *P. cystidiosus* mushroom was soaked in 1000 mL of ethanol pro-analysis solution for 60 minutes. The immersion was then put into a shaker incubator at 40-50 rpm for 8 hours at 65 ^oC, followed by sonication using a sonicator for 3 hours at 70 ^oC at a frequency of 50 Hz for 3 cycles. After sonication, shaking was carried out with a shaker incubator for 24 hours, and then the simplisia was filtered. The total macerate obtained was then evaporated with a vacuum rotary evaporator at 70 ^oC with a speed of 50 rpm until a thick extract was obtained. The thick extract obtained is then evaporated using an evaporator chamber until a dry preparation is obtained.

2. Freeze Granule Preparation (Freeze-Drying)

Prepare frozen granules of P. cystidiosus using freeze-drying equipment (VIRTIS: BENCHTOP 2K XL). The evaporated *P. cystidiosus* thick ethanol extract was put into a freezer until the extract froze (turned into ice crystals). This was done to speed up the evaporation process. In the next stage, the thick extract already in the freeze dryer is analyzed and tested with a temperature setting of -50 ^oC. This freeze-drying process will take place with different temperature variations at 36 hours, 48 hours, and 72 hours, with the final result being dry powder ready for use. The dry freeze-dried *P. cystidiosus* granules were evaluated by looking at the condition of the container where the granule preparation was no longer cold when touched by hand. The solution tested for antibacterial was made by dissolving the frozen granule of *P. cystidiosus* extract with Analisis Fourier Transform Infrared (FTIR). FTIR analysis was conducted to obtain information about the compounds in *P. cystidiosus* frozen granule samples more quickly, non-destructively, and simply. The infrared wavelength used in this identification is 4000 - 400 cm^{-1}. The purpose of doing it at these wavelengths is to find out the absorption spectrum of each sample, and through the chemometric principle, the sample will be analyzed. Frozen garnet samples were analyzed for three repetition cycles to obtain more diverse data from the chemometric analysis process. Chemometric analysis provides a better advantage because it can draw as much data as possible about a chemical compound present in the sample and facilitate reading.

3. Antibacterial Testing of *P. cystidiosis* Against 4 Pathogenic Bacteria

Antibacterial testing of frozen P. cystidiosus granules was carried out against four types of pathogenic bacteria, namely Listeria monocytogenes ATCC 7664, Pseudomonas aeruginosa ATCC 9027, Escherichia coli ATCC 8739, and Staphylococcus aureus ATCC 25923. Testing was carried out using the microdilution method with the concentration of P. cytisus granules used, namely 125 ppm (K1), 250 ppm (K2), 500 ppm (K3), and 1000 ppm (K4). Testing was conducted within 24-36 hours, with observations every 2 hours. IC_{50} calculations were carried out for all experimental groups of pathogenic bacteria used.

4. Data Analysis

Data obtained from FTIR testing is then analyzed descriptively analytically by comparing pick data and chromatograms formed with existing databases and IR standard tables. Antibacterial test data were analyzed using SPSS software version 27.0 and GraphPad Prism Ver. 10.3.0 with 95% confidence level. The analysis results are presented as mean ± standard error data on the histogram of visualization results with GraphPad Prism.

C. Results

The frozen granule compounds of *P. cystidiosus* extract were identified using Fourier-transform infrared spectroscopy (FTIR). The results of FTIR analysis are presented in the chromatogram image (Figure 1). The reading of absorbance values and spectra at a wavelength of 500 - 3500 nm showed a chromatogram pick and a variety of IR absorption. The IR absorption value for the C-H bonding alkene group is 2800 - 2900 cm^{-1}, while the O-H group has the largest distribution in the 3000s range (3000 - 3500 cm^{-1}). The IR absorption results of P. cystidiosus granule samples show IR absorption in the 400 - 3300 cm^{-1} range, with the highest value at 3204.15 cm^{-1} and the lowest at 426.79 cm^{-1} (Figure 1). The reading results by comparing the FTIR identification data on the sample with the reference, and the IR table obtained five classes of compounds that are very likely to be contained in frozen granules of *P. cystidiosus* fruit body extract, namely tannins, alkaloids, flavonoids, saponins, and steroids (Table 1).

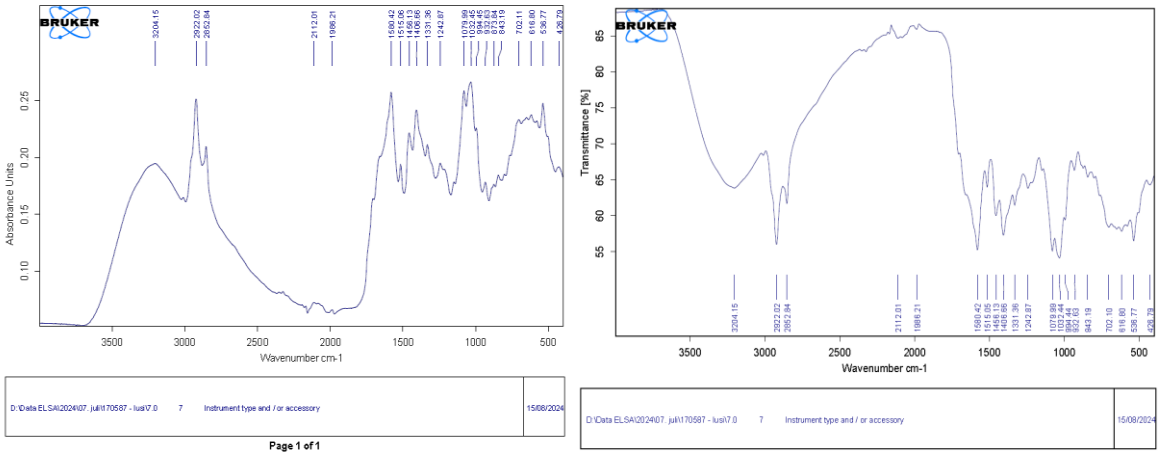
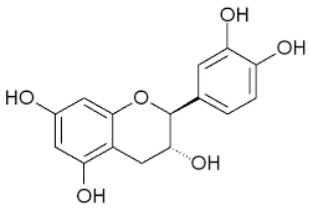
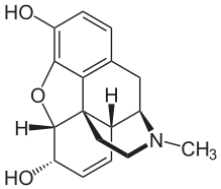
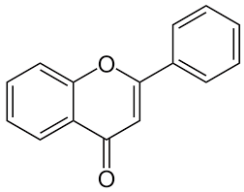
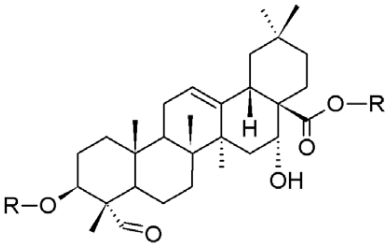
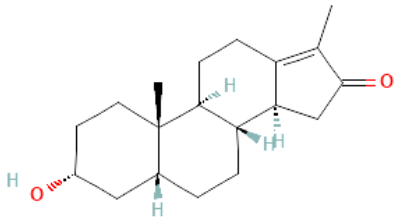


Figure 1. Chromatogram FTIR *P. cystidiosus* granules

Table 1 shows the bioactive compounds in *P. cystidiosus* granules with IR absorption values of 1000 - 3000 cm^{-1} (1331.36 - 3204.15 cm^{-1}). The most easily identified compound is the O-H group, which has an absorption range above 3000 cm^{-1} and a curved and widened chromatogram pick shape (Figure 1).

The tightest pick with an IR absorption value of 400 - 1000 cm^{-1} may be included in groups such as alkyl, nitro compounds, and aryl halides such as C with IR absorption values > 1000 cm^{-1} and C < 600 cm^{-1} (Figure 1).

Table 1. FTIR reading result of frozen granule of *P. cystidiosus* ethanol extract

No	Wave Numbers (cm^{-1})	Functional Groups	Supporting Secondary Metabolites	Compound Structure
1	3200 – 3500 3204.15	-OH Alcohol	Tannins	
2	2800 – 3000 2852.84 – 2922.02	NH Amina	Alkaloids	
3	1500 – 1900 1580.42 – 1986.21	-C=C	Flavonoids	
4	1300 – 1500 1331.36 – 1515.06	-Ketone	Saponins	
5	2800 – 2900 2852.84	-CH Alkana	Steroids	

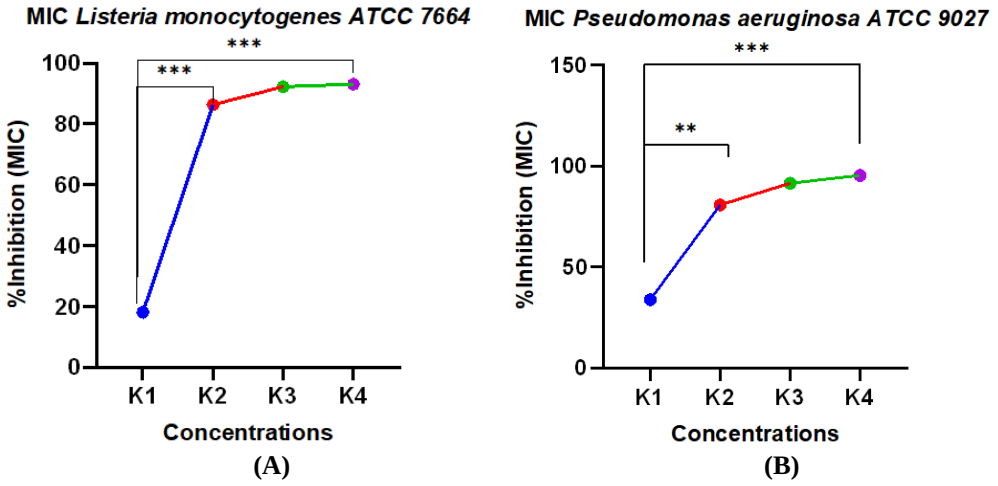
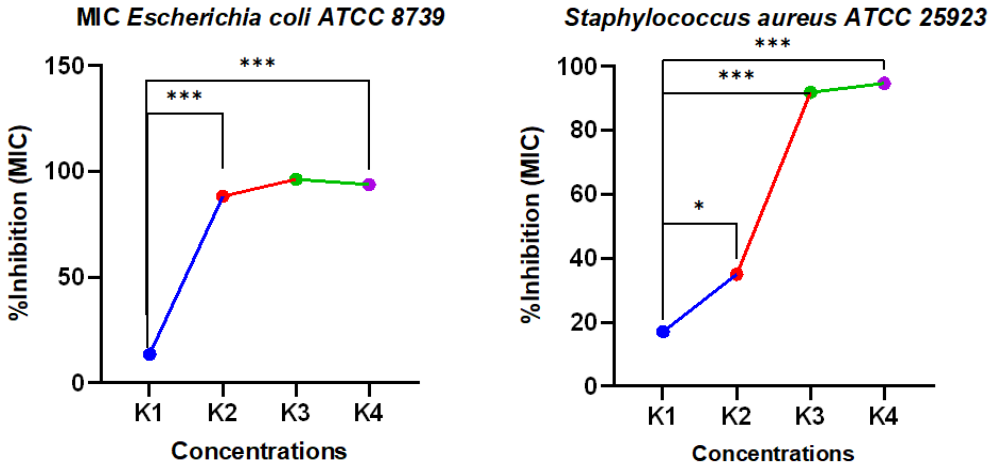


Figure 2. MIC of *Listeria monocytogenes* ATCC 7664 (A) and *Pseudomonas aeruginosa* ATCC 9027 (B); K1: 125 ppm concentration, K2: 250 ppm concentration, K3: concentration of 500 ppm, and K4: concentration 1000 ppm

Based on the results of MIC testing against *L. monocytogenes* ATCC 7664 and *P. aeruginosa ATCC 9027* bacteria, four concentrations of *P. cystidiosus* granules showed different and highly significant values (*p < 0.05; ****) (Figure 2). The test results against *L. monocytogenes* ATCC 7664 (gram-positive) bacteria showed that the administration of *P. cystidiosus* granules in group K4 gave the highest results with a MIC inhibition percentage value of 96.25%, followed by K3 with 93.10%, K2 88.27%, and the lowest K1 with 18.14% (Figure 2A). This also shows that the higher the concentration of granule administration, the higher the MIC value obtained. The same thing was also shown in the gram-negative bacteria testing group, namely *P. aeruginosa ATCC 9027*, where the administration of *P. cystidiosis* granules with the highest concentration of 1000 ppm (K4) gave the highest MIC inhibition value of 95.28%, but was not significantly different from the K3 group with a MIC value of 91.49%, while in the K2 group it was 80.65%, and K1 only 33.75% (Figure 2B).



(A) **(B)**

Figure 3. MIC of *Escherichia coli* ATCC 8739 (A) and *Staphylococcus aureus* ATCC 25923 (B); K1: 125 ppm concentration, K2: 250 ppm concentration, K3: concentration of 500 ppm, and K4: concentration 1000 ppm

Testing of frozen granules of *P. cystidiosus* extract was also carried out against other gram-positive and negative bacteria, namely *E. coli ATCC 8739* (gram-negative) and *S. aureus ATCC 25923* to obtain further information regarding the effectiveness of this brown oyster mushroom granule. The test results against these two bacteria showed varying values, while the analysis results showed highly significant values (*p < 0.05 ****) (Figure 3). Testing using *E. coli ATCC 8739* bacteria showed that the frozen granule of *P. cystidiosus* extracts with the highest MIC inhibition value was group K3 with 96.25%. In contrast, group K4 with 93.80%, K2 with 88.27%, and group K1 had smallest MIC value with 13.65%. There was a decrease in the MIC value in group K4 with a granule suspension concentration of 1000 ppm, compared to K3 with a concentration of 500 ppm (Figure 3A). Different results were shown in MIC testing against gram-positive bacteria *S. aureus ATCC 25923*, where group K4 had the highest inhibition value at 94.67%, K3 at 91.88%, K2 at 35.05%, and K1 at 17.03% (Figure 3B). Overall, the frozen granule test of *P. cystidiosus* extract can inhibit the growth of gram-positive and gram-negative bacterial isolates.

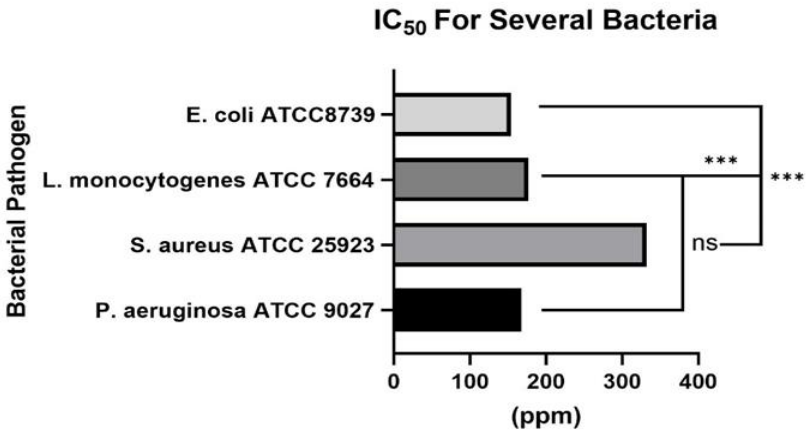


Figure 4. IC_{50} Value for Escherichia coli ATCC 8739, Listeria monocytogenes ATCC 7664, Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 9027 (MIC: ppm)

Based on the measurement and calculation of the Inhibition Concentration (IC_{50}) value of the four isolates used, it shows that the administration of frozen granules of *P. cystidiosus* extract, in general, has the potential to inhibit the growth of the bacteria used, where the statistical test results show highly significant results ($p < 0.05$ ***), and non-significant for *P. aeruginosa ATCC 9027* (Figure 4). The IC_{50} value shows the effectiveness of the frozen granule of *P. cystidiosus* extract; the lower IC50 value of the *P. cystidiosus* granule against microorganisms shows its effectiveness and ability to kill microbes is improving. Based on the calculation results, the highest IC_{50} value was found in the experimental group using *S. aureus* ATCC 25923 bacteria with an IC_{50} value of 335.76, followed by the experimental group using *L. monocytogenes ATCC 7664* bacteria with an IC_{50} value of 176.94, the experiment using *E. coli ATCC 8739* bacteria with an IC_{50} value of 178.59, and the experimental group with the lowest IC_{50} value was *P. aeruginosa 9027* bacteria with 171.65.

D. Discussion

Based on the extraction results, 21.08 grams of thick extract was obtained from the fruiting body of *P. cystidiosus* using pro-analys ethanol solvent. The extraction was carried out using the sonication method, where the results of sonication optimization at a frequency of 45-50 Hz with three cycles. The process of making frozen granules of *P. cystidiosus* fruit body extract was carried out at a temperature setting of -40 ^{o}C, with a maximum temperature of 80 ^{o}C. The sensor was HM4100 with a pressure level of 2-4 mbar. The results of freeze drying obtained 15.67 grams. The reading results of frozen granule samples of *P. cystidiosus* fruit body extract using FTIR showed diverse and complex results. Absorbance readings with a wavelength of 500 nm showed IR absorption values of more than 400 $cm^{-1,}$ with maximum absorption values identified at more than 3000 cm^{-1}. These results show that IR absorption in mushroom granule samples is complex and varied. These results are difficult to interpret and read. Previous research that identified compounds in Mentha piperita extract also showed the complexity of the results on the pick chromatogram of FTIR results with absorption values >300 cm^{-1}. In addition, previous studies have shown that the absorption value >3000 cm^{-1} is included in the NH-amine and -OH alcohol groups, with the results of identifying secondary metabolite compounds as alkaloids and tannins. The IR absorption value for the wave number of flavonoid metabolite compounds is 1500-1700 cm^{-1}, with the functional group being -C=C aromatic, while the -CO ketone functional group with secondary metabolites identified as saponins shows IR absorption values at wave numbers above 1600 cm^{-1} [4]. The results of this study show that the absorption of IR values with wave numbers ranging from 1300 - 3500 cm^{-1} are steroid

compounds, saponins, flavonoids, alkaloids, and tannins, with the highest tannin absorption above 3200 cm^{-1}.

Bioactive compounds in *P. cystidiosus* granules are very important to be identified and characterized to obtain information on their potential and benefits as antibacterials so that further isolation of single compounds can be carried out to be developed into an antibacterial supplement product or herbal medicine candidate [5]. Flavonoid compounds are a group of -OH groups with chemical structures and abundant hydroxyl content, as well as tannins. Compounds included in the phenolic group have great potential as antibacterials and antioxidants because they can disrupt the permeability of pathogenic bacterial cell membranes and are toxic to bacteria [6]. Steroid compounds in an organic substance present in the extract, if given singly to cancer cells or groups of pathogenic bacterial cells, can also be toxic (cytotoxic) [7]. The cytotoxic activity of this group of bioactive compounds is needed in pathogenic bacterial cells so that their growth decreases, does not infect, and prevents bacteria from reproducing further. Previous research also showed that the compounds in Pleurotus mushrooms are terpenoids, alkaloids, and phenolics tested qualitatively. Terpenoid compounds can induce apoptosis in pathogenic cells. At the same time, phenolic compounds with -OH groups or without -OH groups also have antibacterial activity by suppressing bacterial growth and damaging bacterial cell membranes [8].

Based on the results of MIC testing against four bacterial isolates *E. coli ATCC 8739*, *L. monocytogenes ATCC 7664*, *S. aureus ATCC 25923*, and *P. aeruginosa ATCC 9024*, it has shown that the frozen granule suspension of *P. cystidiosus* fruit body extract is effectively able to inhibit the growth of the four isolates. The MIC values of the four bacterial isolates that illustrate the effectiveness of growth inhibition after the administration of *P. cystidiosus* granules after 18-24 hours also provide information that the bioactive compounds contained in *P. cystidiosus* granules have the potential to be developed as antibacterial/antibiotic candidates. The highest inhibition was generally shown against *P. aeruginosa* and *E. coli* bacterial groups. Previous research showed that the *P. ostreatus* mushrooms could inhibit the growth of *E. coli and S. aureus* bacteria with an inhibition value of 90.86% and 92.67%, respectively [9]. Evaluation with the MIC method using the macro-broth dilution technique has been widely developed to evaluate the effectiveness of an extract of natural ingredients such as mushrooms. Previous research also showed that the use of the MIC macro-broth dilution method using *P. pulomarius* mushrooms with various concentrations was able to inhibit the growth of bacteria *S. mutans, S. aureus, P. vulgaris, P. aeruginosa, Klebsiella pneumoniae, E. coli,* and *Candida tropicalis*. The highest MIC values were 23.3 (*E. coli*) and 21.7 (S. mutans). In general, *P. pulmonaris* mushroom extract can inhibit the growth of gram-positive and gram-negative bacteria, and the results show that the inhibition against gram-negative bacteria is higher than that of gram-positive bacterial species [10]. This is following this study, where the best average MIC value of the four doses used (concentrations of 125, 250, 500, and 100 ppm) is in the experimental group of gram-negative bacteria (*E. coli ATCC 8739* and *P. aeruginosa* ATCC 9027), while the lowest average MIC value results from the four doses used in the *S. aureus* ATCC 25923 bacteria experiment.

It is known that mushrooms bioactive compounds that can be isolated from extracts such as phenolic compounds, flavonoids, carotenoids, polysaccharides, steroids, organic acids, steroids, and alkaloids have potent activity in inhibiting the growth of gram-positive or negative bacteria [10]. This also follows the research results showing that *P. cystidiosus* contains several compounds identified in the previous study. Previous research that tested the inhibitory activity of *P. ostreatus* extracts with the disc method also showed that *P. ostreatus* extracts containing tannins, flavonoids, alkaloids, and steroid compounds were able to inhibit the growth of *E. coli*, *Shigella* sp., *Staphylococcus* sp., *Vibrio* sp., and *Peniclillum* sp. with the formation of the largest inhibition zone against *E. coli* bacteria (gram-negative) with 19 mm and *Staphylococcus* sp.

with 10 mm [11]. Meanwhile, the results of inhibitory activity evaluated by IC_{50} values also showed that the IC_{50} values for *E. coli ATCC 8739* and *P. aeruginosa ATCC 9027* (gram-negative bacteria) were lower when compared to the group of gram-positive bacteria used. It is known that the smaller the IC_{50} value generated from a substance or test result, the higher the substance's effectiveness in inhibiting the tested bacteria's growth [12]. Previous research also showed that Moringa leaf extract containing flavonoids can inhibit the growth of E. coli bacteria, with the highest inhibition zone value of 27.7 mm [13].

The development of natural-based antibiotics needs to be improved and further developed, considering the many bacterial resistances found in the treatment using synthetic antibiotics on the market today. In addition, natural substances have less risk of side effects if used in the long term when compared to synthetic drugs. β-glucan polysaccharide compounds are the most common and dominant compounds developed as antibacterial substances, in addition to their activities as antioxidants and immunomodulators [14]. β-glucan in brown oyster mushroom (*P. sajor-caju*) contains quite a lot, with more than 30% of total β-glucan, and contains more than 2.30 g for phenolic compounds [15]. Other studies have also shown that bioactive compounds in ethanol extracts of *P. ostreatus* fruiting bodies can inhibit the growth of *E. coli* bacteria with an inhibition zone of 27 mm, *Salmonella* sp. with an inhibition zone of 20 mm, and *S. aureus* bacteria with an inhibition zone of 23 mm. Based on these results, it can generally be concluded that the ethanol extract of *P. ostreatus* is widely able to inhibit the growth of gram-positive and negative bacteria [16]. When compared with other mushrooms, the species group of the Pleurotus genus, such as (*P. pulmonaris* and *P. ostreatus*) contains more antibacterial compounds (flavonoids) than *Agaricus bisporus* with flavonoid content in *P. ostreatus* 12.02 mg/mL and *P. pulmonaris* 16.44 mg/mL, while in *A. bisporus* only 10.22 mg/mL. Still, the total phenolic *A. bisporus* is more [3].

E. Conclusions

The results showed that the frozen granule contained bioactive compounds of tannins, alkaloids, flavonoids, saponins, and steroids. The frozen granule of *P. cystidiosus* fruiting body extract inhibited the growth of pathogenic bacteria *E. coli, S. aureus, L. monocytogenes,* and *P. aeruginosa*. The best inhibition was found at concentrations of 500 and 1000 ppm for each test group, while the best IC_{50} value was found in the gram-negative bacteria experimental group (*E. coli ATCC 8739* and *P. aeruginosa ATCC 9027*). *P. cystidiosus* has the potential to be developed as an antibacterial that can inhibit the growth of gram-positive or negative pathogenic bacteria.

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