

Antibacterial and cytotoxic activities of supernatant and mycelium extracts from fermentation of fungal symbiont *Trichoderma reesei* TV221

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ABSTRACT

Fungi are microorganisms that can be in a symbiotic relationship with marine sponges in producing secondary metabolites. One of the sponge-associated fungi that have been studied and have antibacterial and cytotoxic activity is *Trichoderma reesei* TV221. This fungus comes from the *Stylissa flabelliformis* sponge from Menjangan Island, West Bali National Park, Bali. This study aimed to determine the growth curve of fungi fermented with seawater made from natural sea salt with a concentration of 30 parts per trillion (ppt), to be tested for antibacterial and cytotoxic activities on the mycelium and supernatant components of the fermentation, and to determine the selectivity index (SI). The growth curve was plotted by determining the dry weight of the mycelium every 2 days for 14 days of fermentation. The antibacterial activity of the extract was tested against *Escherichia coli* American Type Culture Collection (ATCC) 25922 and *Staphylococcus aureus* ATCC 25923, while the cytotoxic activity was tested against the WiDr cancer cell line and Vero cell line. The results showed that the maximum growth of *T. reesei* TV221 fungi took place on day 12. The mycelium and supernatant extracts from the fermentation had a potential antibacterial activity, especially against *S. aureus* ATCC 25923, especially fermentation on days 6 and 12. The supernatant extracts on days 12 and 14 had potential cytotoxic activity against the WiDr cancer cell line and had an SI value > 3. Based on the antibacterial and cytotoxic activities, it is likely that the active metabolites of the *T. reesei* TV221 fungus began to be produced on day 2 and reached their peak on day 12. The active metabolites contributing to the antibacterial and cytotoxic activities were estimated to be cyclohexanone, phenol, and terpenoid groups.

INTRODUCTION

Marine sponges are marine organisms from the phylum Porifera whose life is in symbiosis with various microbes, including bacteria, cyanobacteria, microalgae, and fungi. The microbial symbiosis in some marine sponges can reach 60% of their body weight and contributes significantly to the metabolism

of these sponges (Brinkmann *et al.*, 2017; Taylor *et al.*, 2007). The association between symbionts and marine sponges has enormous potential in health science, especially in the discovery of new medicinal substances derived from natural products. The results of combining marine sponges with symbionts as secondary metabolites demonstrate potential bioactivity as an antimicrobial or antibacterial agent, antitumor agent, cytotoxic agent, inhibitor of cell division, and inhibitor or enzyme inhibitor (Suman *et al.*, 2013; Thoms *et al.*, 2007). Several active metabolites, such as jasplakinolide, bryostatin, and microluside A, seem to be produced by microbial symbionts found in marine sponges (Egan *et al.*, 2008; Eltamany *et al.*, 2014; Rungprom *et al.*, 2008).

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As a result, symbionts derived from microbial sponges can be cultured and isolated to increase the production of some sponge-derived metabolite compounds (Lee *et al.*, 2001). Fungi are microorganisms that can work together with sponges to produce secondary metabolites. In order to survive in these conditions, the sponge association function will produce secondary metabolites which are probably bioactive compounds for medicines. It is necessary to investigate the possibility of increasing the number of medicinal products derived from marine resources (Debbab *et al.*, 2011; Shalini *et al.*, 2014).

One of the sponge-associated fungi that have been researched and have antibacterial and cytotoxic activity is *T. reesei* TV221 (Setyowati *et al.*, 2017, 2018a, 2018b). This fungus is a sponge-associated fungus from the *Stylissa flabelliformis* marine sponge from Menjangan Island, West Bali National Park. The marine sponge *S. flabelliformis* was previously known to have antimicrobial and cytotoxic activities (Setyowati *et al.*, 2005; Setyowati *et al.*, 2021). The extract from the fermentation of the *T. reesei* TV221 fungi associated with the *S. flabelliformis* sponge has antimicrobial activity against *Staphylococcus aureus* American Type Culture Collection (ATCC) 29213, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, and methicillin-resistant *S. aureus* (Setyowati *et al.*, 2017). The extract also has cytotoxic activity on cancer cell lines WiDr, 4T1, T47D, and Raji, with the best cytotoxic activity on WiDr cells, and has the highest selectivity index (SI) compared to the Vero cell line (normal cells) (Setyowati *et al.*, 2018a, 2017). According to the results of previous studies, the fungus *T. reesei* TV221 associated with the sponge *S. flabelliformis* could be developed as an antibacterial and anticancer agent.

In previous research, only the supernatant extract from the fermentation of the fungus *T. reesei* TV221 was tested for antibacterial and cytotoxic activities. In this research, antibacterial and cytotoxic activity tests were carried out on the mycelium components from the fermentation of the *T. reesei* TV221 fungus and the growth curve of fungi fermented with seawater made from natural sea salt with a concentration of 30 ppt (3%) was determined.

MATERIALS AND METHODS

Materials

Trichoderma reesei TV221, a fungal symbiont from the *S. flabelliformis* sponge, was from Menjangan Island, West National Park, Bali. Also used were sabouraud dextrose agar (SDA) (Oxoid) and sabouraud dextrose broth (SDB) (Oxoid). Microbial tests used in the study were *E. coli* ATCC 25922, *S. aureus* ATCC 25923, nutrient agar (NA) (Oxoid), and nutrient broth (Oxoid). WiDr cancer cells line and Vero cells line were sourced from the Cancer Chemoprevention Research Center Universitas Gadjah Mada. Other materials used were MTT solution (Sigma-Aldrich), phosphate-buffered saline (PBS) (Sigma-Aldrich), fetal bovine serum (Sigma), penicillin-streptomycin (Gibco, Invitrogen Canada), Roswell Park Memorial Institute 1640, trypsin-EDTA (Gibco, Invitrogen Canada), sodium dodecyl sulfate (SDS) (Merck Schuchardt, Germany), 5-fluorouracil (Sigma-Aldrich), and Microplate 96 (IWAKI).

Equipment

The equipment used included the following: inverted microscope (Olympus®, Japan), CO₂ incubator (Heraeus®, Germany), Cytotoxic Safety Cabinet (ESCO®, type Cytoculture),

Haemocytometer (Assistet®), vacuum evaporator (Heidolph®), microplate reader (Bio-Rad®, Japan), and. ELISA reader (ELx, BioTech®).

Cultivation, fermentation, and extraction of fungal metabolites

The *T. reesei* TV221 fungal isolate associated with the *S. flabelliformis* sponge was used in a previous study and stored at the Indonesian Center for Biodiversity and Biotechnology Bogor for recultivation (Setyowati *et al.*, 2018b). The fungal isolates were transferred to the SDA medium. A Petri dish was incubated at ambient temperature for 7 days (Setyowati *et al.*, 2017). To produce secondary metabolites, fermentation was carried out with a liquid medium. The fungus was cultivated at 25°C for 14 days using SDB dissolved in seawater with a salinity of 30 ppt (30 g of natural sea salt, 1,000 ml of distilled water) to as much as 200 ml for each fermentation flask and placed on a rotary shaker (120 rpm). Fermentation sampling was carried out every 2 days, followed by the separation of the supernatant and mycelium. The mycelium obtained was dried at 55°C for 3 days, followed by determining the dry weight of the mycelium, and then sonicated with ethyl acetate for 30 minutes three times. The supernatant was carried out by liquid extraction with ethyl acetate and the organic part was taken. The extract obtained from the supernatant and mycelium was then evaporated by rotary evaporation at 60°C until dry and the extract was then weighed.

Antibacterial activity

The bacteria used for the study were *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 of which the concentration of bacteria was 10⁷ CFU suspended on a 10 ml NA medium. Ethyl acetate extracts from the supernatant and mycelium were made with a concentration of 10 mg/ml with methanol and 10 µl of each was sprayed into a sterilized paper dish. As a positive control, chloramphenicol 1 mg/ml was used. As much as 10 µl was sprayed into a sterilized paper dish. Each paper dish was placed on NA solid media which already contained a bacterial suspension. The Petri dish was incubated overnight at 37°C. The inhibition zone that formed around the paper dish was observed (Niyomkam *et al.*, 2010).

Cytotoxic activity on WiDr Cancer cell line and Vero cell line

Cancer cells were prepared in a concentration of 10⁴ cells for each well in 96-well plates. The cell line was incubated for 24 hours in a 37°C CO₂ incubator. Following incubation, the medium was removed and washed using 100 µl PBS solution/well. Samples with seven variations of concentration (7.81, 15.62, 31.25, 62.5, 125, 250, and 500 µg/ml) and 5-fluorouracil control were put into the 96-well plates, each of which was 100 µl/well. It was incubated again in a 37°C CO₂ incubator for a period of 48 hours. Samples and control were removed, the well was washed using 100 µl PBS/well twice, and then a 100 µl/well MTT solution was then added to each well and incubated for 4 hours in a 37°C CO₂ incubator. The formation of formazan crystals was observed under an inverted microscope. After the formazan crystals were formed, a 100 µl/well stopper reagent (10% SDS in 0.1 N HCl) was added and incubated at room temperature in the dark overnight. The cell density was determined by absorbance reading using an ELISA

reader at a wavelength of 550 nm (Cancer Chemoprevention Research Center, 2019; Janakiraman *et al.*, 2020; Setyowati *et al.*, 2018a; Sharmalkumar *et al.*, 2020). The percentage of viable cells was calculated by the following formula:

$$\text{Percentage of viable cell (\%)} = \frac{(\text{Abs sample} - \text{Abs media})}{(\text{Abs normal} - \text{Abs media})} \times 100$$

Calculation of SI

The SI is the value of IC_{50} in a normal cell line/ IC_{50} in a cancer cell line, where IC_{50} is a concentration capable of killing 50% of the cell population. The sample under study was said to have high selectivity if it had a value of $SI \geq 3$ (Sutejo *et al.*, 2016). The SI was determined by the following formula:

$$SI = \frac{IC_{50} \text{ on normal cell (Verso Cells)}}{IC_{50} \text{ on cancer cell (WiDr Cells)}} \times 100$$

RESULTS AND DISCUSSION

Trichoderma reesei TV221 is a fungal symbiont of the *S. flabelliformis* marine sponge obtained from Menjangan Island, North Bali. The results of the fermentation of the *T. reesei* TV221 fungus on SDB medium with seawater for 11 days were known to have antibacterial and cytotoxic activity against several cancer cell lines (Setyowati *et al.*, 2017, 2018a, 2018b). However, previous

studies neither revealed when the secondary metabolites began to be produced nor determined the salinity level of the seawater used. To determine these, in this study, in obtaining the secondary metabolites from the *T. reesei* TV221 fungus, the fungal culture was fermented in liquid media of SDB with seawater made from natural sea salt with a concentration of 30 ppt and fermented samples were taken every 2 days, followed by the determination of antibacterial and cytotoxic activity on the WiDr cancer cell line.

The life phase of the fungus can be described by the growth curve observed from sampling every 2 days during fermentation. The growth curve of *T. reesei* TV221 is shown in Figure 1 (a). *Trichoderma reesei* TV221 passes through the log phase on days 4–10, while the stationary phase starts from days 10 to 12 which is followed by a declination phase from day 12. The highest dry weight of mycelium was produced on day 12 (1,062 mg). Fungi and microorganisms in general will produce secondary metabolites in the stationary phase. The fungus associated with the *Cinachyrella* sp. sponge, *T. reesei*, was reported to have a growth curve similar to the stationary phase that occurs around day 12 (Sibero *et al.*, 2018). In previous studies, *T. reesei* showed the highest antibacterial activity in the fermentation on days 11–12 (Setyowati *et al.*, 2017). The nutrient content of the fungal growth media in solid and liquid greatly affects the growth of fungi. In producing bioactive metabolites, a fermentation medium is essential. Sources

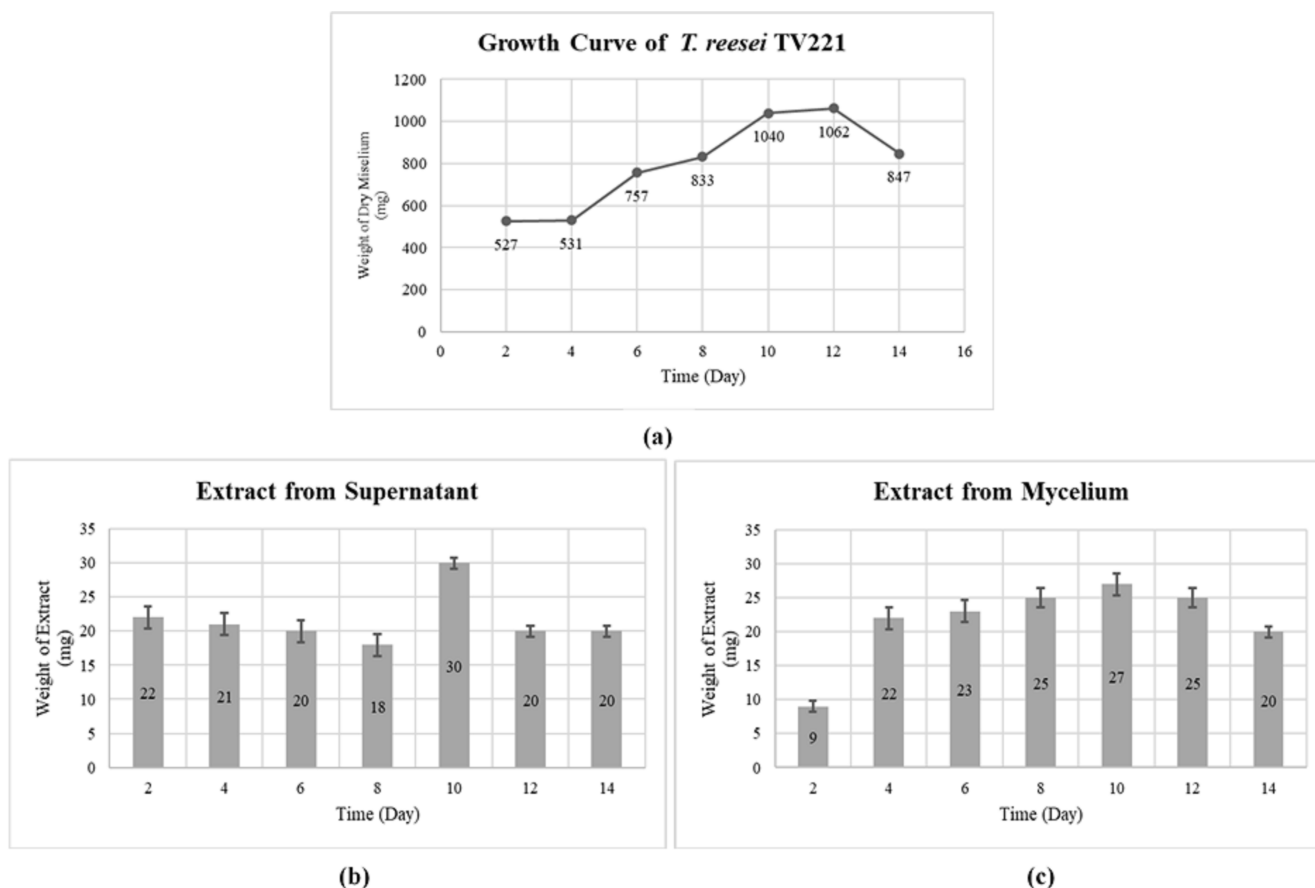


Figure 1. (a) Growth curve of fungal *T. reesei* TV221; (b) Weights of the extract from the supernatant; and (c) Weights of the extract from mycelium.

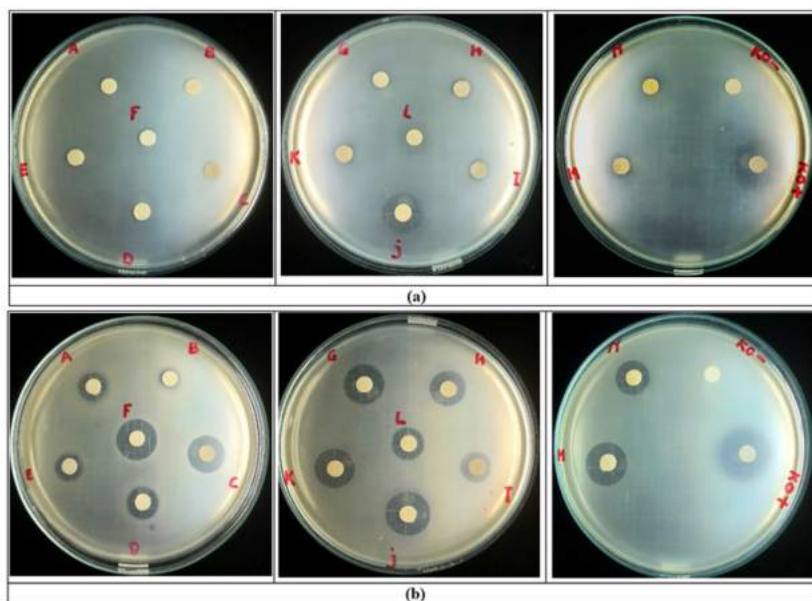


Figure 2. Antibacterial effect of the extracts from mycelium (sample codes A–G) and supernatant (sample codes H–N); (KO–) = negative control; (KO+) = positive control. (a) *Escherichia coli* ATCC 25922; (b) *Staphylococcus aureus* ATCC 25923.

of carbon, nitrogen, and salinity are factors in the production of bioactive metabolites from marine sponge symbionts, especially fungi (Samirana *et al.*, 2020; Ukhty *et al.*, 2017). In Figure 1 (b) and (c), the weight of the extracts derived from the supernatant and mycelium resulting from the fermentation is presented. The highest weight of both supernatant and mycelium extracts occurred on day 10 of fermentation. This is possible because the *T. reesei* TV221 fungus entered a stationary phase where the fungus began to maximally produce secondary metabolites (Stanbury *et al.*, 1995).

In previous research, the *T. reesei* TV221 fungus was known to have antibacterial activity on *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 (Setyowati *et al.*, 2017, 2018b). The samples of the study were only the supernatant extract from the fermentation results and the fermentation was carried out with seawater whose concentration was unknown. In this study, the tests were carried out on the supernatant and mycelium extracts with a seawater concentration of 30 ppt. The results of the antibacterial activity test of the supernatant and mycelium extract fermented on days 2–14 are shown in Figure 2. The potential antibacterial activity of the supernatant and mycelium extracts was shown against *S. aureus* ATCC 25923 as a representation of Gram-positive bacteria starting on day 2 of the fermentation. The highest antibacterial activity of the supernatant extract against *S. aureus* ATCC 25923 occurred on days 6 and 12 of the fermentation, while that of the mycelium extract occurred on days 6 and 12 of the fermentation. This shows that the antibacterial active components were released from the fungal cells maximally on days 6 and 12 into the supernatant and maximally accumulated in the fungal cells on days 6 and 12. Previous research also showed the same phenomenon, in which the highest antibacterial activity against *S. aureus* from the supernatant extract of *T. reesei* TV221 fungi occurred on days 6 and 12 of the fermentation (Setyowati *et al.*, 2017). The antibacterial activity against *E. coli* ATCC 25922 from the supernatant and mycelium extracts from the fermentation of

T. reesei TV221 fungi with seawater at a concentration of 30 ppt showed lower results than the activity against *S. aureus* ATCC 25923. The mycelium extracts only showed activity against *E. coli* ATCC 25922 on days 10 and 12 of the fermentation, while the supernatant extracts showed the highest activity against *E. coli* ATCC 25922 on day 6 of the fermentation. The antibacterial activity of the supernatant extract on day 6 is in line with the results of previous studies (Setyowati *et al.*, 2017). The reduced antibacterial activity of the supernatant and mycelium extracts from the fermentation of the *T. reesei* TV221 fungus against *E. coli* ATCC 25922 as a representation of Gram-negative bacteria is possible because the salinity levels were different from previous studies. Several species of the genus *Trichoderma* fungi were reported to have high sensitivity to environmental salinity, which can affect the growth and production of secondary metabolites resulting in biological activity (Bheemaraya *et al.*, 2013; Mishra *et al.*, 2016; Samirana *et al.*, 2020).

The diameter of the inhibition zone in the antibacterial test against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 can indicate the ability of the sample to inhibit microbial growth. An overview of the diameter of the inhibition zone is shown in Figure 3. The widest diameter of the inhibition zone in the study for the antibacterial activity against *E. coli* ATCC 25922 was shown by the supernatant extract on day 6 (14.33 mm), while the narrowest one was shown by the mycelium extract on days 10 and 12 (6.67 mm). The mycelium extract on days 2–8 and 14 and supernatant extracts on days 8 and 10 did not show any inhibition zone against *E. coli* ATCC 25922 bacteria. This shows that the supernatant extract was more active than the mycelium extract, where the supernatant extract from days 6 to 12 of the fermentation had the potential as an antibacterial agent against *E. coli* ATCC 25922, which represented Gram-negative bacteria. The widest diameter of the inhibition zone in the antibacterial test against *S. aureus* ATCC 25923 was shown by the supernatant extract on day 6 (17.67 mm) and the narrowest one was shown by the mycelium

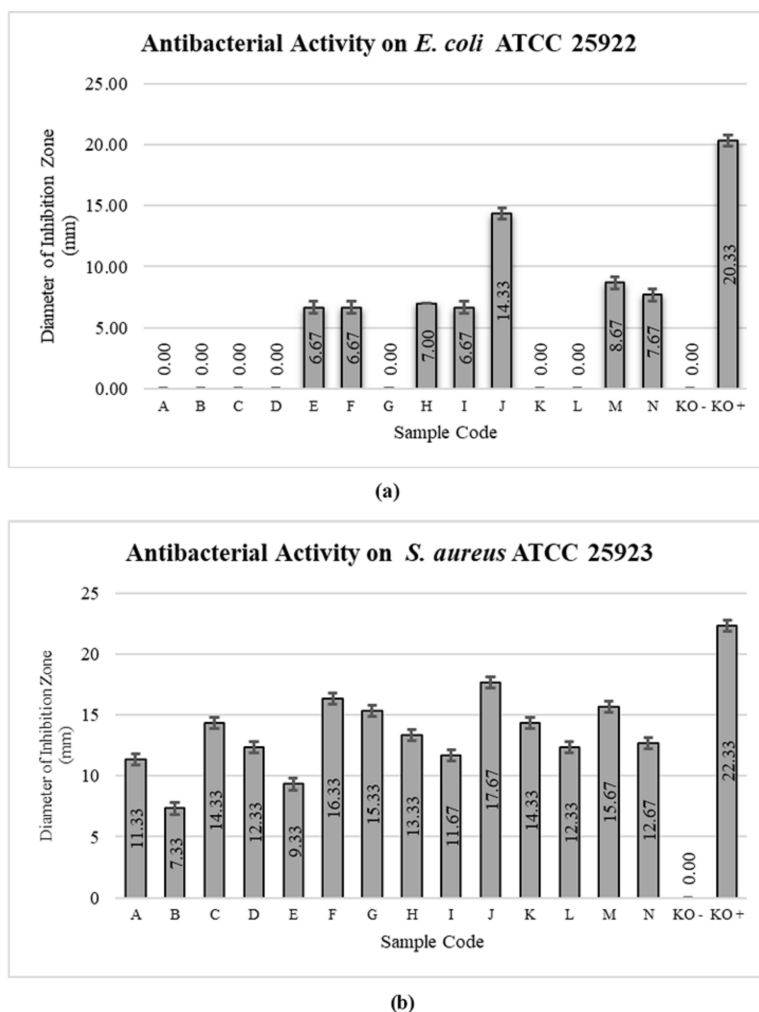


Figure 3. Value of the antibacterial inhibition zone diameter at (a) *Escherichia coli* ATCC 25922 and (b) *Staphylococcus aureus* ATCC 25923. Extracts from mycelium (sample codes A–G) and supernatant (sample codes H–N); (KO–) = negative control; (KO+) = positive control.

extract on day 4 (7.33 mm). All the supernatant and mycelium extracts showed antibacterial activity on *S. aureus* ATCC 25923 which represented Gram-positive bacteria. The mycelium extract on days 12 and 14 and the supernatant extract on days 6 and 12 had a wide inhibition zone diameter, so these extracts had the potential as an antibacterial agent against *S. aureus* ATCC 25923. Chloramphenicol, an antibiotic with a broad spectrum, was used as a positive control, which showed an inhibition zone diameter against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 of 20.33 mm and 22.33 mm, respectively. The criteria for the inhibition zone diameter for antibacterial activity are as follows: inhibition zone diameter of 5 mm or less is categorized as having weak antibacterial activity, inhibition zone of 5–10 mm is categorized as having moderate antibacterial activity, inhibition zone of 10–20 mm is categorized as having strong antibacterial activity, and inhibition zone of 20 mm or more is categorized as having very strong antibacterial activity (Davis *et al.*, 1971a, 1971b). Based on these criteria, the mycelium extracts on days 10 and 12 and the supernatant extracts on days 12 and 14 against *E. coli* ATCC 25922 were classified as having moderate antibacterial activity, and the supernatant extract on day 6 was classified as having strong antibacterial activity. The

antibacterial activity against *S. aureus* ATCC 25923 from all the mycelium and supernatant extracts was strong, except the mycelium extracts on days 4 and 10 which were classified as moderate. The extract activity was shown to be more active against *S. aureus* ATCC 25923 compared to *E. coli* ATCC 25922 because *E. coli* ATCC 25922 which are Gram-negative bacteria have a cell wall structure that is more complex than *S. aureus* ATCC 25923 which are classified as Gram-positive bacteria (Pelczar *et al.*, 2008a; Sangavi *et al.*, 2019; Shahid *et al.*, 2015). In the positive control, the inhibition zone was classified as having very strong antibacterial activity. In fact, this is natural considering the fact that chloramphenicol is a broad-spectrum antibiotic, so it is effective for both Gram-negative and Gram-positive bacteria (Tjay *et al.*, 2015).

The type of inhibition zone in the antibacterial test using a disc can indicate the sensitivity of bacteria to antibacterial agents or antibiotics as test materials. The type of inhibition zone can be divided into a radical zone and an irradiational zone. The radical zone type is indicated by a clear boundary between the bacterial colony and the clear zone that is not covered by the bacterial colonies, and this is usually called the kill zone type. The irradiational zone

Table 1. Inhibition of antibacterial activity on *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

Sample code	Specimen	Inhibition type	
		<i>Escherichia coli</i> ATCC 25922	<i>Staphylococcus aureus</i> ATCC 25923
A	Mycelium day 2	N.A	Radical
B	Mycelium day 4	N.A	Irradical
C	Mycelium day 6	N.A	Radical
D	Mycelium day 8	N.A	Radical
E	Mycelium day 10	Irradical	Radical
F	Mycelium day 12	Irradical	Radical
G	Mycelium day 14	N.A	Radical
H	Supernatant day 2	Irradical	Radical
I	Supernatant day 4	Irradical	Radical
J	Supernatant day 6	Radical	Radical
K	Supernatant day 8	N.A	Radical
L	Supernatant day 10	N.A	Radical
M	Supernatant day 12	Irradical	Radical
N	Supernatant day 14	Irradical	Irradical
KO–	Negative control	N.A	N.A
KO+	Positive control	Irradical	Irradical

N.A: Not active.

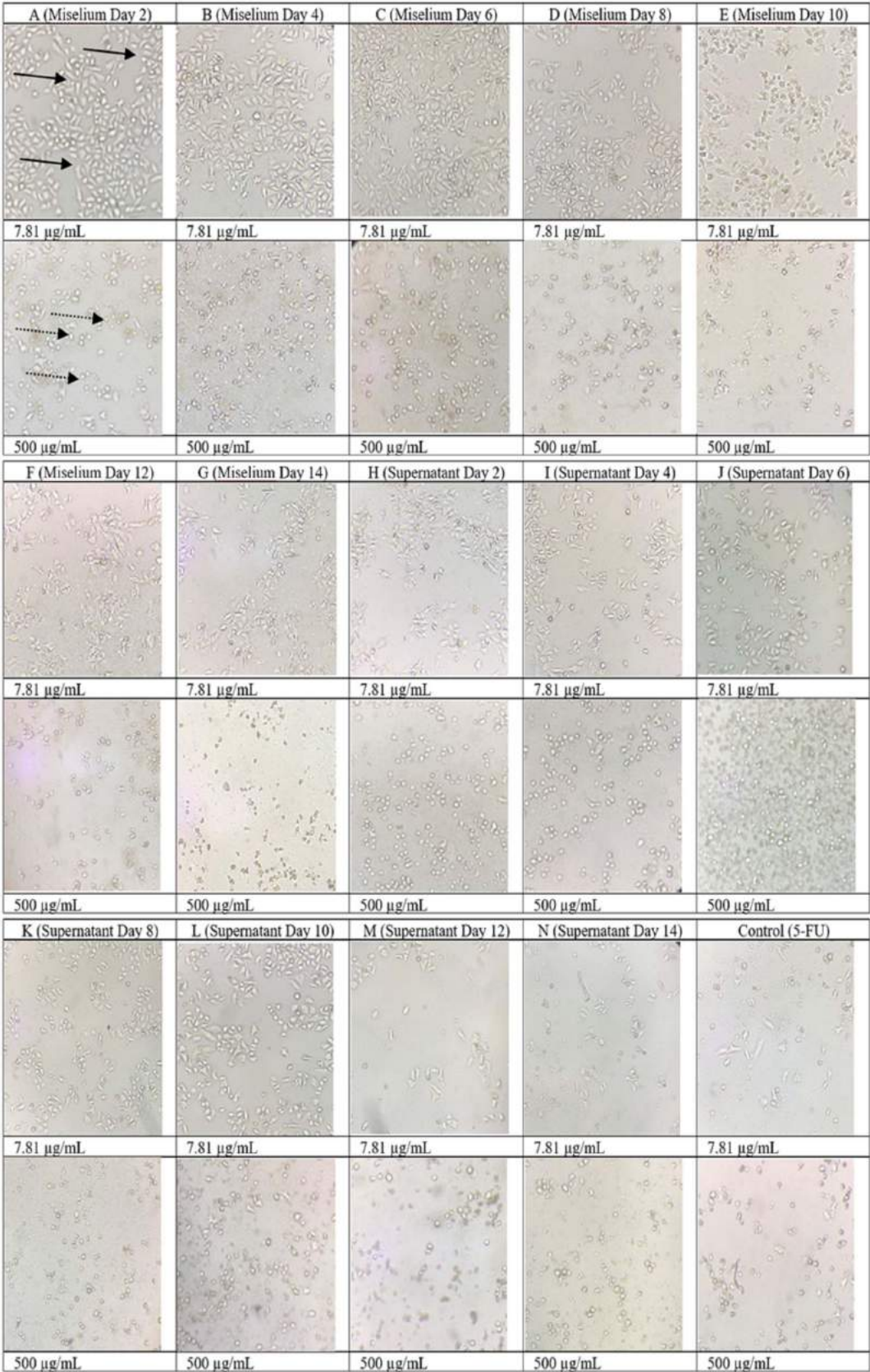
type is indicated by the presence of unclear or equal boundaries between the bacterial colony and the clear zone with bacterial overgrowth, usually referred to as the inhibition zone type (Pelczar *et al.*, 2008a, 2008b; Balouiri *et al.*, 2016). The type of the inhibition zone of the mycelium and supernatant extracts in the antibacterial test is presented in Table 1. Most of the inhibition zone against *E. coli* ATCC 25922 was of the irractical type and only one sample of the extract was categorized as the radical type in the supernatant extract on day 6. Therefore, the extract which is active as an antibacterial agent against *E. coli* ATCC 25922 has the potential to inhibit bacterial growth as seen from the type of inhibition zone. Most of the inhibition zone in the antibacterial test against *S. aureus* ATCC 25923 was categorized as the radical type and only two extract samples of the mycelium extract on day 4 and the supernatant extract on day 14 were categorized as the irractical inhibition zone type. Therefore, the extract which is active as an antibacterial agent against *S. aureus* ATCC 25923 has the potential to kill bacterial growth as seen from the type of inhibition zone.

The results of the cytotoxic test of the mycelium and supernatant extracts on the WiDr cell line are shown in Figure 4, which showed the morphological photos of the WiDr cancer cell line given the treatment at the highest (500 µg/ml) and the lowest (7.81 µg/ml) concentrations. In the cell morphology image, in general, at the highest dose of treatment, the cancer cell line died which was marked by a round cell shape not attached to the surface. Treated at the lowest dose, the WiDr cancer cell line morphology looked like epithelial cells that were dilated and attached to the surface base, indicating the cell line was still alive (Fadholly *et al.*, 2019; Proboningrat *et al.*, 2021).

Cytotoxic activity is indicated by the value of the IC₅₀ sample, which is the result of the calculation of the linear regression equation where the *x*-axis is the log of concentration and the *y*-axis is the percentage of cell viability. The smaller the value of IC₅₀, the

higher the potential of the sample to have cytotoxic activity. The largest IC₅₀ value of the mycelium extract was on day 6 (5,654.58 µg/ml) and the smallest one was on day 14 (205.63 µg/ml). The supernatant extract had the largest IC₅₀ value on day 2 (255.61 µg/ml) and the smallest on day 12 (81.72 µg/ml). The IC₅₀ value of the extract of natural ingredients can be classified into three levels of cytotoxic activity; namely, IC₅₀ value < 10 µg/ml has a very strong cytotoxic effect, IC₅₀ value 10–100 µg/ml has a strong cytotoxic effect, and IC₅₀ value of 100–500 µg/ml has a moderate cytotoxic effect (Samirana *et al.*, 2020; Weerapreeyakul *et al.*, 2012). Based on the data presented in Table 2, generally the mycelium and supernatant extracts had cytotoxic activity on the WiDr cancer cell line which was classified as a moderate cytotoxic effect, except for the supernatant extract on days 12 (81.72 µg/ml) and 14 (81.93 µg/ml) which had strong cytotoxic activity. The control (5-fluorouracil) had an IC₅₀ value of 24.99 µg/ml, which had strong cytotoxic activity on the WiDr cell line. 5-Fluorouracil is known to be the first line in the treatment of colon cancer, so this study used it as the control (Masi *et al.*, 2006). The IC₅₀ value of the supernatant extract on the WiDr cancer cell line on day 12, which was 81.72 µg/ml, was almost similar to that of the previous study which reported that the IC₅₀ value of the supernatant extract against the WiDr cancer cell line was 88.88 µg/ml, as the result of the fermentation of *T. reesei* TV221 fungi for 11 days (Setyowati *et al.*, 2018a).

The results of the cytotoxic test on the Vero cell line of the four extracts and positive control (5-FU) are shown in Figure 5, which showed the morphological photos of the Vero cell line given the highest (1,000 µg/ml) and lowest (15.62 µg/ml) concentrations. The higher concentration of sample will decrease the absorbance value which depicts the metabolic activity of living cells which is decreased. A decreased value of absorbance with increasing concentration of sample shows an increase in the number of cells that die and decreasing of number of live cells (Dwiandhono *et al.*, 2020;



→ : Living Cells ;→ : Death Cells

Figure 4. The Morphology of The WiDr Cancer Cell Line When Given The Treated Samples at Doses of 7.81 µg/mL and 500 µg/mL

Liu *et al.*, 2011). In the cell morphology image, on average, at the highest dose of treatment, the Vero cell line died, marked by cell shrinkage, loss of cell nuclei, and not adhering to the surface. In the treatment at the lowest dose, the morphology of the Vero cell line looked like a dilated epithelial cell. The cell nucleus could be observed and attached to the base surface indicating that the cell line was still alive (Ammerman *et al.*, 2008). The use of the Vero cell line aimed at seeing the effect of the sample on normal cells to determine whether the test substance could damage normal cells or not. Based on the data presented in Table 3, the IC₅₀ value of the four extracts and the positive control (5-FU) had moderate cytotoxic activity against the Vero cell line. This shows that the

cytotoxic activity of the four extracts and the control on Vero cells did not cause severe damage to normal cells.

The SI value was determined by comparing the IC₅₀ value in the Vero cell line with the IC₅₀ value on the WiDr cancer cell line. The results of the SI values of the four extracts and the control are presented in Table 4. The sample was said to have high selectivity against a cancer cell if SI value ≥ 3 (Badisa *et al.*, 2009; Sutejo *et al.*, 2016). The four extracts and the control (5-FU) had an SI value > 3 , meaning that the four supernatant extracts and 5-FU had high selectivity against WiDr cancer cells, so in the future it is very likely that they are developed as anticancer agents for colon cancer.

Referring to the antibacterial and cytotoxic activity of the supernatant and mycelium extracts, the active metabolites of *T. reesei* TV221 fungus began to be produced on day 2 of the fermentation as indicated by their biological activity. Active metabolites began to reach their peak production starting on day 6 and the peak occurred on day 12, indicated by the maximum antibacterial and cytotoxic activity. Therefore, *T. reesei* TV221 can be said to reach the highest active metabolites and activity. *Trichoderma reesei* TV221 should be fermented for 12 days. This is in line with the growth chart for the fungus which peaked on day 12 and then declined. In the growth phase of microorganisms, the peak of the stationary phase before the decline phase is the optimal state for a microorganism to produce active metabolites (Stanbury *et al.*, 1995; Pratiwi, 2008).

Based on previous research, the metabolite groups found in the *T. reesei* fungus which is in symbiosis with the *S. flabelliformis* marine sponge are the cyclohexanone, phenol, and terpenoid group (Setyowati *et al.*, 2017). This group of compounds has been shown to have a potential antimicrobial activity (Liu *et al.*, 2013). In addition, the *S. flabelliformis* marine sponge has been known to produce jaspamide/jasplakinolide which has antibacterial and cytotoxic activity (Himaya *et al.*, 2013; Liu *et al.*, 2009; Setyowati *et al.*, 2004, 2005). Many studies have linked the active compounds

Table 2. IC₅₀ values of the sample on the WiDr cancer cell line.

Sample code	Specimen	IC ₅₀ (µg/ml)
A	Mycelium day 2	448.37
B	Mycelium day 4	671.83
C	Mycelium day 6	5,654.58
D	Mycelium day 8	746.67
E	Mycelium day 10	366.45
F	Mycelium day 12	262.05
G	Mycelium day 14	206.51
H	Supernatant day 2	255.61
I	Supernatant day 4	205.63
J	Supernatant day 6	139.68
K	Supernatant day 8	154.29
L	Supernatant day 10	231.19
M	Supernatant day 12	81.72
N	Supernatant day 14	81.93
Control	5-Fluorouracil	24.99

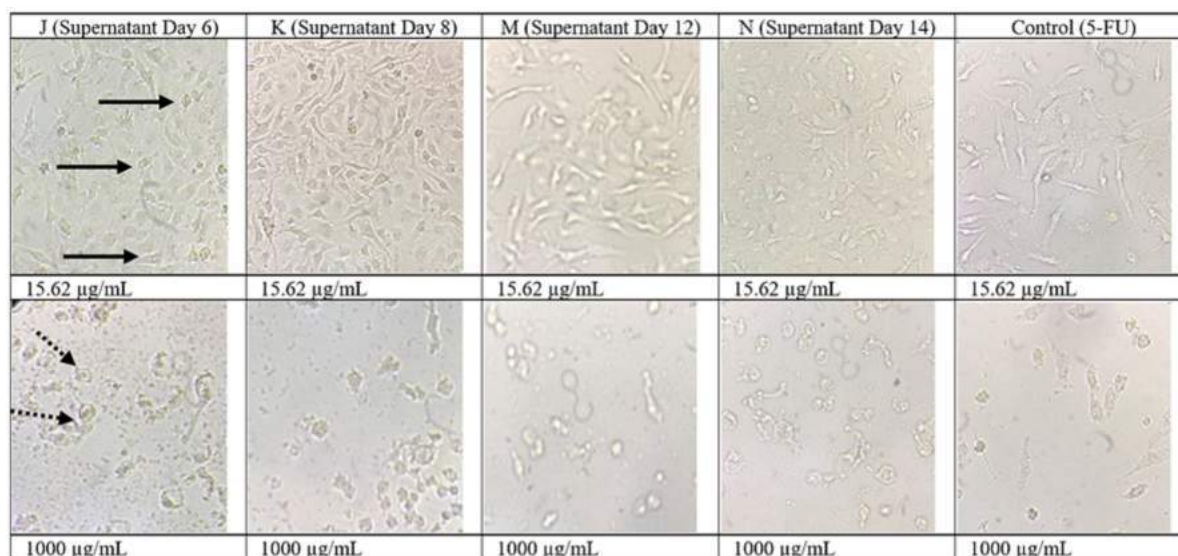


Figure 5. Morphology of the Vero cell line when given the treated samples at doses of 15.62 µg/ml and 1,000 µg/ml. —→: living cells;→: dead cells.

Table 3. IC₅₀ values of the sample on the Vero cell line.

Sample code	Specimen	IC ₅₀ (µg/ml)
J	Supernatant day 6	464.76
K	Supernatant day 8	838.96
M	Supernatant day 12	388.47
N	Supernatant day 14	393.30
Control	5-Fluorouracil	391.31

Table 4. SI of cytotoxic activity on the WiDr cancer cell line.

Sample code	Specimen	SI
J	Supernatant day 6	3.33
K	Supernatant day 8	5.44
M	Supernatant day 12	4.75
N	Supernatant day 14	4.80
Control	5-Fluorouracil	15.66

produced by sponges and their symbionts, and some have concluded that these active compounds are produced by symbionts found in marine sponge bodies. There are even some metabolites that have a similar structure between sponges and their symbionts (Hooper *et al.*, 2002). This shows that the symbionts present in the sponge's body play an important role in the production of metabolites produced by marine sponges. Therefore, it is necessary to conduct further research on the antibacterial and cytotoxic activity of the active compounds of the *T. reesei* TV221 symbiont from the *S. flabelliformis* marine sponge.

CONCLUSION

The growth of the *T. reesei* TV221 fungus reaches its peak on day 12 and produces the highest amount of extract on day 10 of the fermentation. The *T. reesei* TV221 fungus has antibacterial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 in both supernatant and mycelium extracts fermented with seawater at concentrations of 30 ppt, especially on days 6 and 12. Supernatant extracts on days 12 and 14 have cytotoxic activity on the WiDr cancer cell line which is classified as strong cytotoxic effect and has high selectivity on the WiDr cancer cell line as indicated by SI value > 3. Based on the activity shown by the supernatant and mycelium extracts, it is likely that the active metabolites of the *T. reesei* TV221 fungus begin to be produced on day 2 and reach their peak on day 12. Active metabolites that have antibacterial and cytotoxic activity are estimated to be the cyclohexanone, phenol, and terpenoid groups. Based on its activities, in the future the extract from the fermentation of the *T. reesei* TV221 fungus has the potential to be developed as an antibacterial and anticancer agent, especially for colon cancer.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of

data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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