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# INHIBITORY ACTIVITY OF INDONESIAN MICROBIAL EXTRACTS AGAINST PROLIFERATION OF DLD-1 COLORECTAL CANCER CELL LINE

## Aktivitas Penghambatan Ekstrak Mikroba Indonesia terhadap Proliferasi Kanker Kolorektal DLD-1

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***ABSTRACT***
*Colorectal cancer (CRC) is the second deadliest cancer in the world. Several anti-cancer agents are currently used for the clinical treatment of CRC. However, toxicity and drug resistance pose significant challenges in CRC chemotherapy. On the other hand, microbe-derived natural products have been explored as a source for the development of anti-cancer therapeutic agents. This study aimed to examine the potential of the microbial library in BioMCC (Biotech Center-BPPT Microbial Culture Collection) as a source for anti-cancer drug discovery. Among the 720 fungal extracts tested, 60 extracts (8.3%) showed inhibitory activity against the proliferation of the colorectal carcinoma DLD-1 cell line, while not affecting Vero cells (African green monkey kidney normal cell line). One of these active extracts was derived from the fungus* Sporothrix *sp. BioMCC-f.T.7716. Although the inhibitory mechanism of this extract against the proliferation of the DLD-1 cell line could not be determined, this study clearly demonstrated the potential use of Indonesian microbial extracts as a source for the discovery of anti-cancer agents.*

***Keywords****: Colorectal cancer, DLD-1, microbes, drug discovery, fungi*

**ABSTRAK**
Kanker kolorektal (CRC) adalah kanker penyebab kematian terbesar kedua di dunia. Beberapa agen anti-kanker digunakan untuk penanganan klinis CRC. Namun, toksisitas dan resistensi obat menjadi tantangan terbesar dalam kemoterapi CRC. Di sisi lain, produk alam dari mikroba telah digunakan sebagai sumber untuk pengembangan agen terapetik anti-kanker. Studi ini bertujuan untuk menguji potensi dari kultur koleksi mikroba di BioMCC (Biotech Center-BPPT Microbial Collection Culture) sebagai sumber untuk penemuan obat anti-kanker. Sebanyak 720 ekstrak kapang yang diuji, 60 ekstrak (8.3%) diantaranya menunjukkan aktivitas penghambatan proliferasi sel kanker DLD-1, namun tidak terhadap sel Vero (sel normal ginjal monyet hijau Afrika). Ekstrak kapang *Sporothrix* sp. BioMCC-f.T.7716 adalah salah satu dari ekstrak aktif tersebut. Walaupun mekanisme penghambatan ekstrak ini terhadap proliferasi sel DLD-1 tidak dapat ditentukan, studi ini dengan jelas mendemonstrasikan potensi galur kapang sebagai sumber untuk penemuan agen anti-kanker.

**Kata Kunci**: Kanker kolorektal, DLD-1, mikroba, penemuan obat, kapang

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## INTRODUCTION

Colorectal cancer (CRC) has been one of the most common malignant tumors worldwide since 1950 and remains the second leading cause of cancer-related mortality, with 916,000 deaths in 2020 (Ferlay et al. 2020). The disease is influenced by different genetic susceptibilities, environmental factors, and dietary habits. Modifiable factors such as obesity, diabetes, alcoholism, smoking, lack of exercise, and a high-fat diet, particularly red meat consumption, are among the environmental factors contributing to the development of colorectal cancer. Consequently, the prevalence of CRC continues to rise (Moehler et al. 2018). Based on projections considering population growth, aging, and human development, the number of new CRC cases worldwide is expected to reach 3.2 million by 2040 (Xi and Xu 2021).

Early detection of CRC commonly involves screening for the formation of colorectal polyps, small benign clumps of cells that develop on the colon lining. Generally, these polyps can be surgically removed before they progress into cancer. Polyps can penetrate the colon and rectum walls by multiple layers. CRCs originate from the innermost layer (the mucosa) and can metastasize to the lymph nodes and bloodstream depending on the extent of invasion (The American Cancer Society 2020).

Chemotherapy is a major treatment modality for CRC (Huang et al. 2019). Since the 1990s, 5-fluorouracil (5-FU)-based chemotherapy has been the cornerstone of CRC treatment (Vodenkova et al. 2020), along with other first-line combinations such as capecitabine, oxaliplatin, and irinotecan (Vodenkova et al. 2020). However, 5-FU has shown toxicity and limited survival benefits (Glimelius et al. 2013). Additionally, drug resistance poses a significant challenge in CRC chemotherapy (Moehler et al. 2018). Various mechanisms contribute to cancer cells' resistance to chemotherapy (Holohan et al. 2013). For example, thymidine phosphorylase (TP), uridine phosphorylase (UP), orotate phosphoribosyl transferase (OPRT), and dihydropyrimidine dehydrogenase (DPD) are involved in the metabolism and breakdown of 5-FU. Studies have shown a correlation between the activity of these enzymes and the sensitivity of CRC to 5-FU. High expression of TP, UP, and OPRT has been associated with susceptibility to 5-FU therapy (Lindskog et al. 2014; Sakowicz-Burkiewicz et al. 2016). Therefore, finding new and safe therapeutic alternatives is a significant challenge (Gazwi et al. 2022), and the search for substances that can slow cancer growth and prevent relapse continues (Sawicka et al. 2022).

Natural compounds derived from various sources are currently employed in cancer treatment (Cragg and Pezzuto, 2016). Approximately 49% of the anti-cancer drugs used in therapy in Europe over the past 70 years were obtained from living organisms or natural material products (Mehta and Kumar Mehta, 2014). Plant-derived anti-cancer drugs include bisindole alkaloids (vinblastine, vincristine, vinorelbine, vinflunine), semi-synthetic epipodophyllotoxins (etoposide, teniposide, etoposide phosphate), taxanes (paclitaxel, docetaxel, cabazitaxel), and campothecin derivatives (irinotecan and topotecan) (Kinghorn et al. 2016). Irofulven, an anti-cancer agent derived from the fungus *Clitocybe illudens*, has been extensively investigated for its potential in cancer chemotherapy (Kornienko et al. 2015). These examples indicate that microbes also serve as an attractive source for the discovery of anti-cancer drugs.

Despite the success of chemotherapy in treating cancer, the limited number of anti-cancer drugs and drug resistance remain major challenges (Chakraborty and Rahman 2012). Drug resistance during chemotherapy can be associated with mechanisms such as drug efflux, genetic factors, growth factors, increased DNA repair capability, and enhanced xenobiotic metabolism. These mechanisms lead to reduced therapeutic efficacy, complicating cancer treatment (Bukowski et al. 2020). Thus, the discovery of novel effective and safe anti-cancer agents is an urgent task to improve the effectiveness of cancer chemotherapy.

Indonesia, with its geographical uniqueness and tropical climate, is recognized as a country with high biological diversity (von Rintelen et al. 2017), making Indonesian microorganisms a potential source for drug discovery (Waluyo et al. 2021). This study aims to evaluate the

potential of Indonesian microorganisms, particularly fungi, as a source for the discovery of anti-CRC agents. We screened 720 fungal extracts derived from fungal isolates deposited in the Biotech-Center BPPT Microbial Collection (currently part of the Indonesia Culture Collection (InaCC) of the National Agency for Research and Innovation) for their specific antiproliferative properties against the colorectal adenocarcinoma (DLD-1) cell line. The selectivity of the active extracts was also confirmed against a normal cell line.

## MATERIALS AND METHODS

### Location and Time

This study was conducted from January 2022 to November 2022 at the Biotechnology Laboratory, National Research and Innovation Agency (BRIN), Science and Technology Park, South Tangerang, Banten, Indonesia.

### Microbial strains

The microbial isolates used in this study were obtained from BioMCC (Biotech Center-BPPT Microbial Culture Collection), which is now part of InaCC (Indonesia Culture Collection) of the National Agency for Research and Innovation of Indonesia (BRIN). The microbial isolates were preserved in 20% glycerol and stored at -80 °C. BioMCC-f.T.7716 was identified as *Sporothrix sp.* based on morphological and molecular identification results (93% similarity of its ITS sequence against the NCBI GenBank database).

### Microbial extract preparation

Fungal isolates were revived from the -80 °C frozen stock on malt extract agar medium and incubated at 28 °C for 1 to 2 weeks. Fungal culture was performed in both liquid and solid medium. For liquid culture, a fungal colony was transferred into 30 mL of medium F (2% rice powder, 1% glucose, 2% soybean meal, 0.1% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$), F15 (3% glucose, 2% glycerol, 1% dextrin, 1% malt extract, 2% yeast extract, 0.1% tryptone, 0.1% $NH_4NO_3$, 0.1% $KH_2PO_4$, pH 6.5), F2 (2% malt extract, 1.1% glucose, 0.22% yeast extract, 0.05% $KH_2PO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.001% $FeCl_3$, 0.000178% $ZnSO_4$, 0.00055% $CaCl_2$), or F4 (0.5% malt extract, 1% glucose, 4% dextrin, 0.05% $KH_2PO_4$, 0.5% polypeptone, 0.5% soybean meal, 0.2% yeast extract, pH 6.0) in a 250 mL Erlenmeyer flask, and incubated at 28 °C using a rotary shaker (220 rpm) for 7 days. The culture broth was extracted with butanol (1:1) and centrifuged at 3000 rpm for 10 minutes. One milliliter of the butanol layer was transferred into a 96-deep well plate and dried up using a vacuum centrifuge. For solid medium, a fungal colony was transferred into 100 mL of liquid medium F and incubated at 28 °C using a rotary shaker (220 rpm) for 7 days before being inoculated into medium SFR1 (10 g water-soaked rice, 1% corn steep liquor) in a 50 mL centrifuge tube. The culture was incubated at 28 °C for 13 days. The culture was then extracted with 10 mL of 50% ethanol and centrifuged at 3000 rpm for 10 minutes. One milliliter of the supernatant was transferred to a 96-deep well plate and dried using a vacuum concentrator. The dried extract was dissolved in 40 µL of DMSO before use and stored at -20 °C for further use.

### Fungal identification

The fungal isolate was grown on potato dextrose agar (PDA) and incubated at 28 °C for 7 days. The morphology of the fungal colony was observed under a digital microscope (Keyence VHX-970F, Osaka, Japan) after staining with lactophenol blue. Fungal identification was performed based on its morphology according to the literature (Sigler et al. 1990; Kwon-Chung and Bennett 1992; de Hoog 1995; Almeida-Paes et al. 2009; Zhou et al. 2014).

### Materials for mammalian cell culture

The colorectal adenocarcinoma (DLD-1) cell line and African green monkey's kidney (Vero) cell line were maintained and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Incubation was carried out at 37 °C and 5% $CO_2$. Cell growth, as well as cell morphology, was observed under an inverted microscope.

### Cell proliferation inhibitory assay

A total of 100 µL of DLD-1 or Vero cell line culture was spread into each well of a 96-well plate with an initial cell number of $1.25 \times 10^5$

cells/well or 5 × $10^4$ cells/well, respectively. The cells were incubated at 37 °C and 5% $CO_2$ for 24 hours. Microbial extract (dissolved in DMSO) was added to each well at a volume of 0.4 µL (250× dilution) and incubated at 37 °C and 5% $CO_2$ for 48 hours. The cells were washed once using 1× PBS and then incubated in 100 µL DMEM (containing 10% cell counting kit-8, Dojindo Laboratories, Kumamoto, Japan) at 37 °C and 5% $CO_2$ for 3 hours. The surviving cells were quantified by measuring the absorbance at 450 nm using a plate reader (Spectramax Paradigm, Molecular Devices, California, USA). The survival rate of the cells was calculated using the following equation:

$$\text{Survival Rate (\%)} = \left| \frac{(A_{450} \text{ of cell culture treated with microbial extract} - A_{450} \text{ of positive control})}{(A_{450} \text{ of negative control} - A_{450} \text{ of positive control})} \right| \times 100$$

where the positive control is a sample without cells, and the negative control is a cell culture treated with 0.4 µL of DMSO. Microbial extracts that showed a cell line survival rate of less than 50% were regarded as a hit.

## RESULTS AND DISCUSSION

### Inhibitory activity of fungal extracts against proliferation of DLD-1 cell line

A total of 720 fungal extracts were prepared and subjected to DLD-1 cell line culture. The survival rate of the DLD-1 cell line in the presence of fungal extract is shown in Figure 1. Among the 720 extracts, 60 extracts (8.3%) showed inhibitory activity against the proliferation of the DLD-1 cell line. The screening was performed using a 96-well plate format with a z'-factor value between 0.98 and 0.89 (average of 0.93) and a signal-to-background ratio (s/b) value between 10.31 and 12.91 (average of 11.53), which is considered an excellent result and the data were reliable (Zhang et al. 1999).

### Secondary screening of hits against Vero cell line

To specifically screen fungal extracts that inhibit the proliferation of the DLD-1 cell line, the hit extracts obtained from the first screening were tested for their inhibitory activities against the proliferation of the Vero cell line, a non-cancer cell line. As shown in Figure 2, among the 60 hit extracts, 11 of them showed little to no effect on the proliferation of the Vero cell line (survival rate more than 50%). The assay was performed using a 96-well plate format with a z'-factor value between 0.64 and 0.95 (average of 0.79) and an s/b ratio value between 7.67 and 10.63 (average of 9.34), which is considered an excellent result and the data were reliable (Zhang et al. 1999).

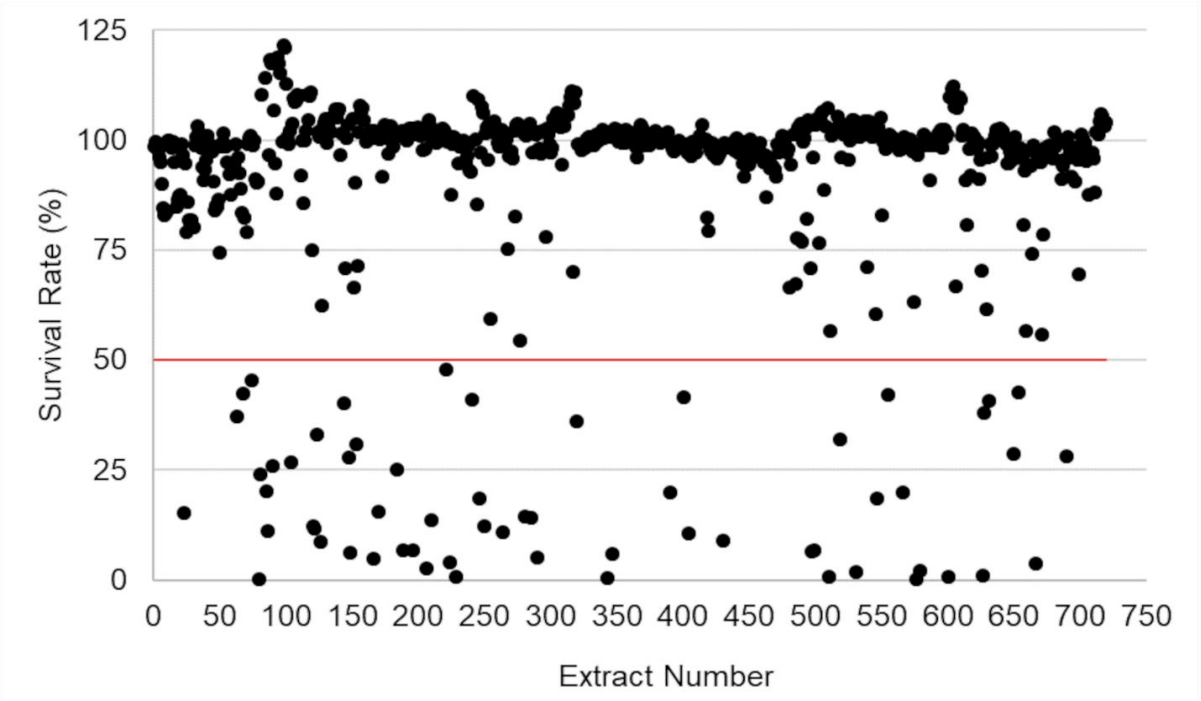


**Figure 1**. Survival rate of DLD-1 cell line after treatment with fungal extracts. Extracts that resulted in a cell survival rate below 50% (indicated by the red line) were considered as hits

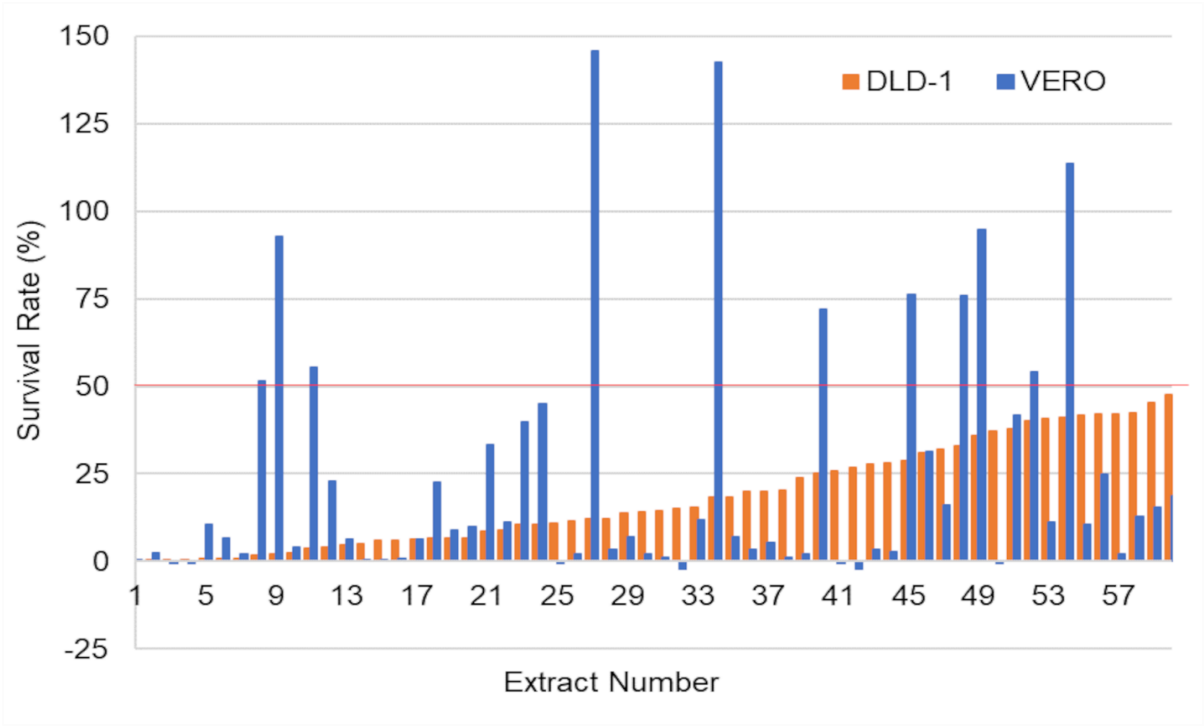


**Figure 2**. Survival rate of the DLD-1 (orange) and Vero (blue) cell lines after treatment with fungal extracts that resulted in a DLD-1 cell survival rate below 50%

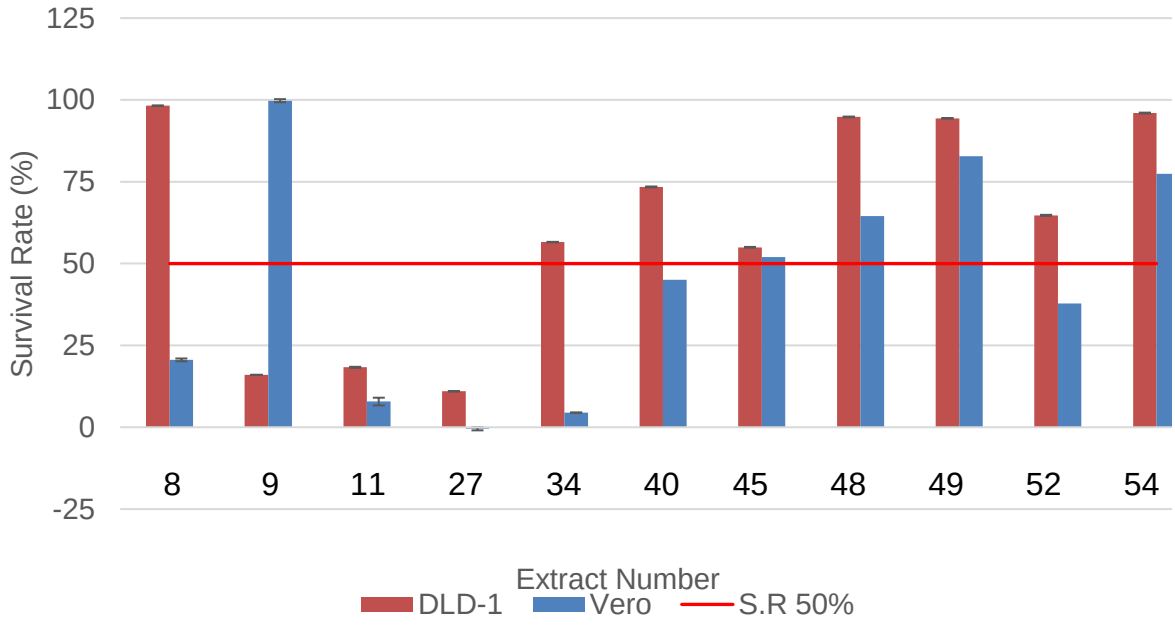


**Figure 3**. Re-assay results of fungal extracts that inhibited the proliferation of the DLD-1 cell line but had little or no effect on the proliferation of the Vero cell line. Orange bars represent DLD-1; blue bars represent Vero; error bars indicate standard deviation (n=3)

## Re-assay of hit extracts

Since microbial extracts occasionally lose their activity during storage (Waluyo et al. 2021), it is important to confirm the stability of active extracts. Eleven hit extracts from the secondary screening were tested for their inhibitory activity against the proliferation of DLD-1 and Vero cell lines after 2 months since being used in the secondary screening. As shown in Figure 3, surprisingly, only 1

extract (extract number 9) inhibited the proliferation of the DLD-1 cell line (survival rate 16%) but did not inhibit the Vero cell line (survival rate 99.7%). The inhibitory activity of this extract was consistent with the results of the first screening (against the DLD-1 cell line) and the secondary screening (against the Vero cell line), indicating that the active compound in the extract was relatively stable during storage. This result also highlights the importance of performing a re-assay of the hits due to the stability issue of microbial extracts during storage. We identified the hit extract as being prepared from a fungal culture of *Sporothrix* sp. BioMCC-f.T.7716.

**Inhibitory mechanism**

Changes in cellular morphology of the DLD-1 and Vero cell lines were observed in the presence of the hit fungal extract. A time-course microscopic observation of the morphological shape of the DLD-1 cell line after the addition of the fungal extract was performed, and the cell morphology was observed at 0 h, 18 h, 24 h, and 42 h after the extract addition. The pictures were compared to those of the Vero cell line in culture treated with the same extract (Figure 4). The morphological shape of the DLD-1 cell changed from a cobblestone shape at 0 h to a spherical shape with observed blebbing starting from 24 h after the addition of the hit fungal extract. In contrast, the morphological shape of the Vero cell did not change after the addition of the extract, suggesting that the extract did not affect the proliferation of the Vero cell. Both cells showed normal growth when DMSO was added to the culture (final concentration of 0.4%), suggesting that DMSO did not affect the morphological changes of both cells.

**Discussion**

According to the BioMCC microbial library database, the active extract was prepared from the culture broth of an Indonesian soil fungus identified as *Sporothrix* sp. BioMCC-f.T.7716 based on its morphology and ITS sequence (93% similarity). It was isolated from Biak, Papua

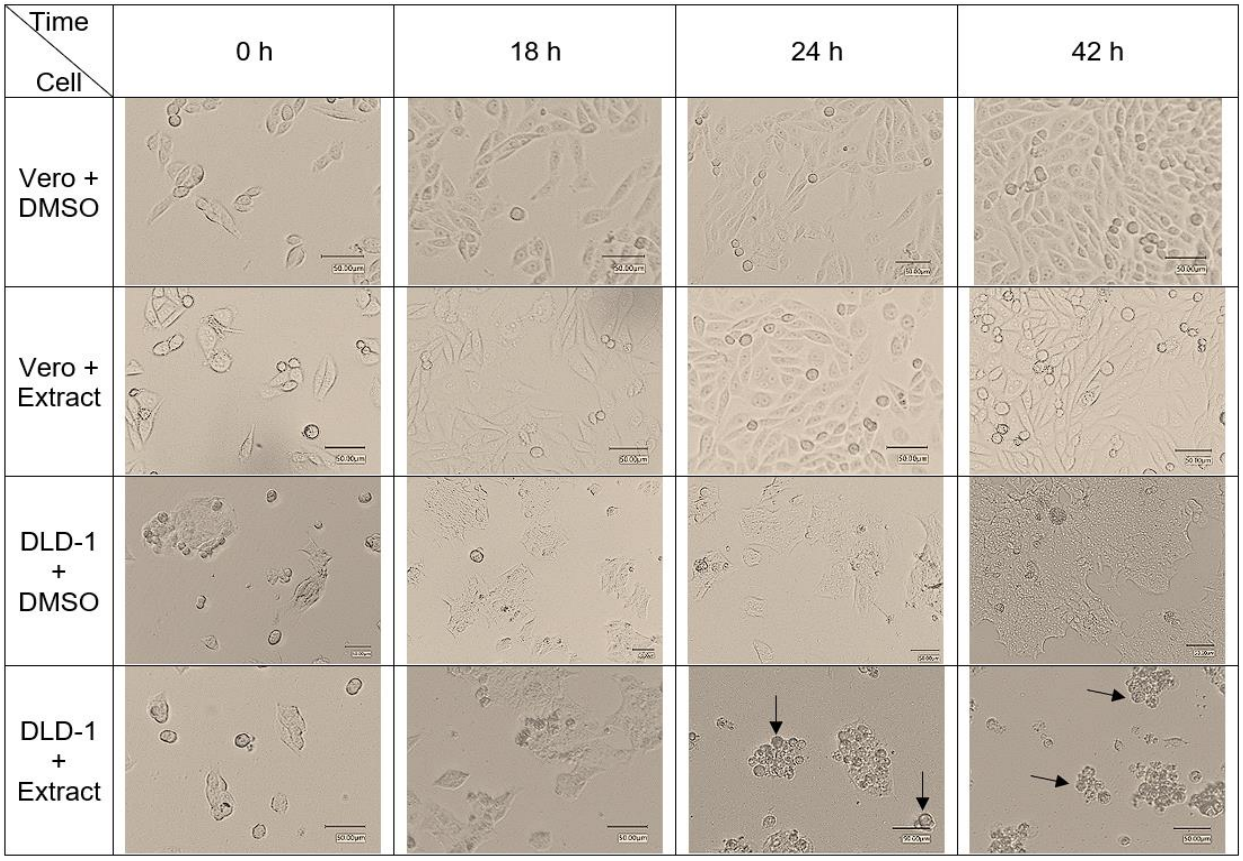


**Figure 4.** Time-course changes in cellular morphology of Vero and DLD-1 cell lines after treatment with DMSO or an extract of the fungus *Sporothrix* sp. BioMCC-f.T.7716. Black arrows indicate blebs (round-shaped dead cells). The bar indicates 25.00µm

Island of Eastern Indonesia, in 2016, using the lithium chloride method (Richter et al. 2008; Mateus et al. 2014). The shape of the fungal colony on PDA medium and the microscopic morphology are shown in Figure 5.

In this study, 720 fungal extracts prepared from Indonesian fungi isolates deposited in BioMCC were tested for their inhibitory activity against proliferation of DLD-1 cell line. From the screening, one fungal extract was obtained that specifically inhibited proliferation of DLD-1 cell line without affecting Vero as a non-cancer cell line. The extract clearly induced cell death in the DLD-1 cell line (Figure 4). Typically, cell death is associated with either apoptosis, a programmed cell death process, or necrosis, an accidental cell death due to environmental perturbations. Several techniques have been developed to discriminate necrosis from apoptosis (Lekshmi et al. 2017), including discrimination based on cell morphology (Yu et al. 2020). However, the toxicity mechanism of the DLD-1 cell line in the presence of the active fungal extract could not be determined based on morphological observation alone, as shown in Figure 4, due to the lack of drugs that have previously been reported to induce apoptosis, such as staurosporine (Lopez et al. 2022), or necrosis, such as cytochalasin B (Hwang et al. 2013), as positive controls. Annexin V is widely used for detecting apoptosis due to its ability to bind with phosphatidylserine, a marker of apoptosis (Abbady et al. 2017). However, further investigation of the toxicity mechanism using this approach was not conducted at the moment. Nevertheless, it will be necessary to characterize the active compound responsible for the inhibitory activity in detail, which will be performed in the near future.

The active extract was prepared from the culture of fungus *Sporothrix* sp. BioMCC-f.T.7716. *Sporothrix* sp. can be found living as a saprophyte and endophyte on living and decaying vegetation, soil, and animal excreta (Rodrigues et al. 2016; Ramírez-Soto et al. 2018), and also in the Antarctic marine habitat (Choi et al. 2022). Generally, *Sporothrix* sp. is known to be harmful to humans as it causes Sporotrichosis. Citromycin, a compound produced by *Sporothrix sp.* isolated from Antarctic marine organisms, was reported to inhibit the migration and invasion of human ovarian cancer A2780 and SKOV3 cells without showing toxicity against them. It also inhibited the EMT (Epithelial–Mesenchymal Transition) markers and the activation of MMP-2 and MMP-9 (matrix metalloproteinase), which are related to metastasis when these proteins are expressed (Choi et al. 2022). Although *Sporothrix sp.* has been reported to produce anti-cancer compounds, no reports have been made regarding a compound from this fungus that specifically inhibits the proliferation of the DLD-1 cell line.

This study demonstrated that the extract of fungal *Sporothrix sp.* BioMCC-f.T.7716 clearly inhibited the proliferation of


**Figure 5**. Morphology of the colony (left, after 7 days of incubation) and conidia (right, stained with lactophenol blue) of the fungus *Sporothrix* sp. BioMCC-f.T.7716 on PDA medium. The bar indicates 25 µm.

the DLD-1 cell line specifically. The search for anti-cancer agents that specifically inhibit the proliferation of cancer cells is important in anti-cancer drug discovery (Zhong et al. 2021). Anti-cancer agents that inhibit the proliferation of cancer cells play a significant role in preventing the spread of cancer cells throughout the body by inducing cytotoxicity, apoptosis, autophagy, and necrosis (Abraham et al. 2017). This can help reduce the size of tumors and make them easier to remove through surgery or other treatments (Varghese et al. 2019). Studying the mechanism of action of the active compound in this extract that is responsible for the anti-cancer activity will provide insight for the development of an effective and safe cancer chemotherapy strategy. This study also suggests the potential of BioMCC as a source for anti-cancer drug discovery.

## CONCLUSION

An active microbial extract was successfully obtained from the culture broth of Indonesian fungal *Sporothrix sp.* BioMCC-f.T.7716, which specifically inhibited the proliferation of the DLD-1 cell line. This finding clearly highlights the importance of microbial resources, particularly microbial isolates deposited in BioMCC, as a source for selective anti-cancer drug discovery. Further isolation and purification of the active compounds responsible for this activity will be the next urgent task to be undertaken.

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