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Soursop leaves (*Annona muricata* L.) endophytic fungi anticancer activity against HeLa cells

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ABSTRACT

Cervical cancer causes many deaths in females worldwide, including in Indonesia. Several studies have reported that soursop (*Annona muricata* L.) leaves can be used to treat cervical cancer. This study aims to determine the use of endophytic fungi of *A. muricata* leaves extract as an ingredient that inhibits cervical cancer. The isolated endophytic fungi from various soursop leaf accessions were grown in culture media, then extracted using ethyl acetate. The extract was then tested against anti-yeast, cervical cancer cells, and on normal cells as control using the MTT method. Five isolated fungi were selected based on the greatest inhibition in one concentration, and the inhibitory concentration 50 (IC₅₀) value was determined. The soursop leaves endophytic fungi extracts showed cytotoxicity against cervical cancer cells by inhibiting the multiplication of HeLa cancer cells *in vitro*. The Sir-SM2 endophytic fungi crude ethyl acetate extract showed high cytotoxicity to cervical cancer cells (HeLa cells) but less harmful to the normal Chang cells; therefore can be a natural anticancer. Identification based on morphology shows that the isolated Sir-SM2 endophytic fungi belong to the *Penicillium* genus, and molecular identification based on Internal Transcribed Spacer shows high similarities with *Penicillium crustosum*.

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1. Introduction

Cervical cancer is a type of cancer that causes the fourth highest death in women with cancer. In 2020, new cases estimated 604,000, with 342,000 deaths worldwide (WHO, 2020a). Cervical cancer can be caused by infection due to excess free radical production, apart from human papillomavirus (HPV) (WHO, 2020b).

Commonly performed treatments for patients with cancer are surgery, chemotherapy, hormone therapy, radiation therapy, targeted drug therapy, and immunotherapy (American Cancer

Society, 2016). Unfortunately, some of these treatments cause side effects, such as causing hair loss, early menopause, weariness, infections, sores on the mouth and throat, and remembrance problems (Mehta and Bhargava, 2019). On the other hand, herbal medicines and phytochemical derivatives have therapeutic effects. Several studies have shown positive results from herbal medicines in improving the quality of life and increasing the immune system of cancer patients (Lee et al., 2018). Additionally, medicinal plants with secondary metabolites showing significant antioxidant activity can play a role in cancer treatment (Seca and Pinto, 2018).

Flowering plants in Indonesia consist of 30,000 species, of which 940 species are identified as having medicinal properties, one of which is soursop. The soursop plant is a type of fruit plant that contains bioactive compounds, such as tannins, phytosterols, flavonoids, saponins, and alkaloids (Agu and Okolie, 2017). Antioxidant compounds prevent the type of free radical damage that has been linked to cancer development (Meng et al., 2020). The soursop plant produces acetogenins that exhibit cytotoxic activity against cancer cells and have special affinities for some resistant cells. Acetogenins from Annonaceae are cytotoxic against several

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cancer and tumor cells, such as pancreatic cancer cells (PACA-2), lung cancer cells (A549), colon, lung, and pancreatic tumors (Nugraha et al., 2019). Research on *Erythrina poeppigiana* leaves showed an inhibitory concentration 50 (IC₅₀) value or inhibition against cervical cancer cells (HeLa) of 7.18 µg mL⁻¹ (Herlina et al., 2019). Bioactive compounds may be produced by endophytic microbes.

Endophytic microbes live in plant tissue by forming colonies without harming the host and producing bioactive compounds that have the potential for drug development (Gouda et al., 2016). Gouda et al. (2016) reported that endophytic microbes could synthesize bioactive compounds similar to those produced by their host due to the evolutionary exchange of genetic information. Metabolite compound production through endophytic microbes will reduce the exploitation of the soursop plant; hence, this plant can continue to produce fruit for public consumption. Minarni et al. (2017) reported that extract of endophytic fungi isolated from the *A. muricata* leaves could suppress the overgrowth of MCF-7 breast cancer cells with an IC₅₀ value of 19.20 ± 7.71 g mL⁻¹. Likewise, Arifni et al. (2017) also reported that soursop leaves endophytic fungi extract has an IC₅₀ value for colon cancer (WiDr) of 20.80 µg mL⁻¹. The endophytic fungi of soursop leaves show significant potential for anticancer activity. Therefore, this study aims to test the endophytic extract activity against cervical cancer cells (HeLa).

2. Material and method

This study used soursop leaves endophytic fungi isolates (from Sukabumi, Cianjur, and Garut), glucose, yeast extract, malt extract, peptone, agar, ethyl acetate, RPMI-1640 (Roswell Park Memorial Institute) cell growth medium, DMEM (Dulbecco's Modified Eagle Medium) cell growth media, doxorubicin, NaHCO₃, aquabides, 96% ethanol (pro-analysis), 100% ethanol (pro-analysis), FBS, penicillin-streptomycin, trypsin, saline phosphate buffer (PBS), cervical cancer cells (HeLa, ATCC[®]-CCL-2[™]), normal cells (Chang, ATCC[®]-CCL[™] 13), Methyl Thyazol Tetrazolium (MTT).

2.1. Media preparation

2.1.1. Malt Glucose Yeast Peptone Agar (MGYPA) medium

Medium of MGYPA was made by dissolving in 1000 mL water, the components such as yeast extract (3 g), malt extract (3 g), glucose (10 g), peptone (5 g), and agar (20 g). The ingredients were put into an Erlenmeyer flask, covered with cotton, then heated in the oven until boiling, and all the ingredients were completely dissolved. Next, the media was autoclaved for sterilization for 15 min at a pressure of 1 atm 121 °C and poured into a Petri dish.

2.1.2. Malt Glucose Yeast Peptone Broth (MGYPB) Medium

Medium of MGYPB was made by dissolving in 1000 mL of distilled water extract of yeast (3 g), extract of malt (3 g), glucose (10 g), and peptone (5 g). All ingredients were put into the Erlenmeyer flask containing 150 mL and covered with modified cotton. The media were sterilized using an autoclave for 15 min at a pressure of 1 atm at 121 °C.

2.2. Endophytic fungi isolate cultivation (Marcellano et al., 2017)

Endophytic fungi isolates originated from the Cianjur region (with accessions codes Sir-CA1, Sir-CA2, Sir-CA3), the Garut region (accessions codes Sir-G2, Sir-G3, Sir-G4, Sir-G5), and the Sukabumi region (accessions codes Sir-SM1, Sir-SM2, Sir-SM3). Endophytic fungi isolates were inoculated aseptically in a special laminar flow. Endophytic fungi isolated from agar slant were aseptically cultured

into MGYPA media and then kept for 2 days at 28 °C ± 2 °C. Secondary metabolite production was conducted by inoculating each endophytic fungi isolate using a tungsten needle into a 150 mL medium of MGYPB and incubated for 21 days at 28 °C ± 2 °C.

2.3. Extraction metabolite compounds (Arifni et al., 2017)

An organic ethyl acetate solvent extracted bioactive compounds from endophytic fungi. Extraction was carried out from endophytic fungi cultured for 21 days at 25 °C. The fungi were cultured in 150 mL of MGYPB medium, supplemented with distillate ethyl acetate, then shaken by hand for 30 min. The fraction at the top layer was then transferred to a heating flask and put in a rotated vacuum evaporator at 36 °C. The extract was dried by simmering of nitrogen gas. The crude extract was kept at 3 °C for further analysis.

2.4. Anticancer activity against cervical cancer cells HeLa with methyl Thyazol tetrazolium (MTT) indicator (Herlina et al., 2019)

The extract was dissolved with RPMI-1640 medium and made a concentration of 100 µg mL⁻¹. The diluted extract was then tested for its activity against HeLa cancer cells. At the first test, the cancer cells without treatment were used as the negative control. For the positive control, 6 µg mL⁻¹ doxorubicin solution was used. The five fungi extract with the best activity according to inhibition percentage were then re-tested against HeLa cancer cells at 25, 50, 100, 200, and 400 µg mL⁻¹ concentrations.

The cells were poured into a sterile conical tube containing RPMI 1640 medium, centrifuged, then a new RPMI 1640 medium was poured into the cell suspension and then recentrifuged until homogeneous. The cell suspension was added with 1 mL RPMI 1640 which have been supplemented with 1% penicillin-streptomycin and 5% FBS. A 100-µL medium-containing cell with a total of 5000 cells/well was added to 96 wells of tissue culture plate and kept for 24 h at 5% CO₂ at 28 °C ± 2 °C to 70–80% confluent. The extract was added as much as 100 µL/well and kept for 48 h, and then washed with PBS and added with 100 µL/well MTT. Cells were kept for 4 h at 5% CO₂ and 37 °C. The supernatant was separated, and 96% ethanol was added. Furthermore, an optical density was measured with an enzyme-linked immunosorbent assay (ELISA) at a wavelength of 595 nm. Data from the ELISA readings are transformed into the inhibition percentage of cells based on the following equation:

$$\%inhibition = \left(\frac{Abs. Control - Abs. Sample}{Abs. Control} \right) \times 100\%$$

Information:

Abs. Control = Absorbance contains no extract.

Abs. Sample = Absorbance of extract solution.

The relationship between cell death and concentration was analyzed using linear regression, and then the extract concentration that could prevent 50% of the cancer cells growth was calculated (IC₅₀). The same procedure was conducted for the cytotoxic activity assay of HeLa cells but using DMEM growth medium added to the tube and kept in the freezer for 24 h.

2.5. Morphological identification of Sir-SM2 (Minarni et al., 2017)

Identification of the Sir-SM2 isolate was conducted by observing the morphological characteristics of the colony and spore. The morphological observation was conducted macroscopically and microscopically, using the identification key (Nithiyaa et al., 2012). The purification results observed Fungi morphology using

a 2-day-old endophytic fungi culture. The medium used for morphological observations was the potato dextrose agar (PDA) medium. Macroscopic observations included colony color and surface (granular, powdery, mountainous, and smooth), texture, growing area, and radial and concentric stripes (especially in *Penicillium* molds). Microscopic observations used a microscope with a magnification of 1000×. The observation was carried out on somatic hyphae type (septate/aseptic), hyphae pigmentation, spore stalk shape, shape, and asexual spore ornamentation (Manan et al., 2017).

2.6. Identification of Sir-SM2 based on amplification of Internal Transcribed Spacer (ITS) (Sundaesan et al., 2019)

The Sir-SM2 isolate was identified molecularly based on partial genetic analysis at the fungi's Internal Transcribed Spacer (ITS) ribosomal deoxyribonucleic acid (DNA) locus. For DNA extraction, fungal isolates were grown in potato dextrose broth liquid medium and incubated at room temperature for 72 h. DNA was isolated from mycelia (Yang et al., 2016) with a modified solution. The hyphae grown on the cellulose membrane were taken and put into a microtube. The mycelium was mashed, and 500 µL of SDS solution was added, which had been incubated in a water bath at 65 °C for 30 min and then quickly transferred to the freezer for 5 min. Chloroform: isopropanol solution of 500 µL was added to the tube, homogenized, and further centrifuged for 10 min at 4 °C 10,000 rpm.

Then the supernatant was taken and put into a new tube. Solution of 500 µL Phenol: chloroform: isopropanol (25:24:1) (v/v) was added to the tube and homogenized. The tube was centrifuged at 10,000 rpm at 4 °C for 5 min, and the supernatant was separated and put into a new tube, added with ethanol absolute, then incubated in the freezer for 24 h. The tube was centrifuged for 30 min at 4 °C 10,000 rpm, then the supernatant was removed, and only the pellet was used for the next step. Approximately 500 µL of 70% ethanol solution was added to the tube and then homogenized. The tube was centrifuged for 10 min at 4 °C 10,000 rpm. The supernatant was discarded, and only the pellet was used. Then, the tube was vacuumed at 45 °C for 30 min. The pellet containing DNA, NFW, and RNase was added to the tube. Before the next step, the concentration and quality of the genomic DNA were determined at wavelengths of 260 and 280 nm using the Nanodrop Spectrophotometer 2000 (Thermo Scientific).

A polymerase chain reaction (PCR) was then conducted to amplify the ITS region of the extracted DNA. Two ITS primers were used, i.e., ITS Primer 4 (reverse): 5'-TCC TCC GCT TAT TGA TAT GC-3', and ITS Primer 5 (forward): 5'-GGA AGT AAA AGT CGT AAC AAG G-3' (Endrawati and Kusumaningtyas, 2021; Ausubel et al., 2004; McBreen et al., 2003). Amplification was conducted on 10 µL consisting of dNTP of 0.2 mM, DreamTaq 1 × buffer (containing MgCl₂), ITS4 Primer of 0.5 pmol, ITS5 Primer of 0.5 pmol, Taq polymerase of 0.125 U, DNA template of 100 ng, and NFW of 5.75 µL. DNA amplification was carried with pre-denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 15 s, attachment at 57 °C for 15 s, and extension at 72 °C for 45 s. The PCR process ended with post-extension at 72 °C for 5 min and cooling at 4 °C for 5 min.

The PCR products were visualized using electrophoresis on 1% agarose gel dissolved in a TAE buffer. Electrophoresis was conducted with a TAE buffer. First, 1 µL of loading dye was mixed with 1 µL of DNA sample and then put into the agarose gel well. All samples were put in gel wells, and then a 2 µL, 1 kb ladder was inserted into one of the wells as a marker well. Samples were electrophoresed with a voltage of 100 V for 30 min. Next, the gel was soaked in EtBr solution for 30 min and then washed with distilled water for 5 min. Finally, the electrophoresis products were

visualized with an ultraviolet transilluminator (Kusumaningrum et al., 2014).

The purification of the PCR results was carried out using PEG and continued with a sequencing cycle. The results of the sequencing cycle were repurified with ethanol according to Sapula et al. (2021). Analysis of nitrogen base sequence readings using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The raw data from the sequencing was then trimmed and assembled using the BioEdit program (<https://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Sequence data that has been assembled is then carried out by Basic Local Alignment Search Tool (BLAST) with genomic data that has been registered at the DNA Data Bank of Japan (<https://blast.ddbj.nig.ac.jp/>) or NCBI/National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/BLAST/>) to define which taxon/species has the greatest homology/similarity and molecularly closest. After that, several organisms were selected to be included in the phylogenetic tree. Percent of homology was calculated using Molecular Evolutionary Genetics Analysis version 5.0 software (MEGA 5) using the Maximum Likelihood parameter. The analysis was carried out with 1000 repetitions using a 2-parameter Kimura model using the Gamma distribution (+G) (Tamura et al. 2011). Monophyletic genera and species were indicated by branches having Bootstrap values (BS) > 80% (Xiao et al., 2020).

3. Results

3.1. Anticancer activity against HeLa cervical cancer cells

The first anticancer activity was tested using one concentration, namely 100 µg mL⁻¹ from ten isolates (Fig. 1). The ten extracts tested at 100 µg mL⁻¹ concentration against cervical cancer cells (HeLa cells) had a good inhibition potential based on the results shown in Fig. 1. The five best extracts were taken from the ten extracts tested based on the highest inhibition percentage (Fig. 1). The best extract was Sir-G2, with an inhibition value exceeding the positive control of 93.63% for the initial test, compared with the positive control (doxorubicin) with 92.85% inhibition.

The five best extracts (Sir-CA1, Sir-CA3, Sir-GA2, Sir-GA4, and Sir-SM2) were then tested at 25, 50, 100, 200, and 400 µg mL⁻¹. The absorbance results of the extract cytotoxic activity inhibition test were then calculated to find the IC₅₀ value. The IC₅₀ value is the concentration of the extract needed to inhibit cancer cell growth by 50% (Nugraha et al., 2019). The IC₅₀ values can be seen in Table 1. Three endophytic fungi extracts (Sir-CA1, Sir-G2, and Sir-SM2) revealed IC₅₀ values of <30 µg mL⁻¹ for HeLa cells. However, Sir-CA1 has the lowest IC₅₀ value but is statistically insignificant ($p < 0.05$) with Sir-G2 and Sir-SM2.

Moreover, extracts with anticancer activity are expected to have low toxic properties to normal cells (selective toxicity) (Cao, 2017). Therefore, the endophytic soursop extracts were tested against Chang's normal cells in our research series and published elsewhere. Sir-CA1 was highly toxic to normal Chang cells, followed by Sir-G2, which produced IC₅₀ values of 8.23 and 23.65 µg mL⁻¹, respectively (Arifni et al., 2017). Additionally, Sir-SM2 had the lowest toxicity activity against Chang's normal cells at 63.69 µg mL⁻¹, compared to extracts from Cianjur (Sir-CA1) and Garut (Sir-G2). Therefore, the Sir-SM 2 was selected for further study.

The anticancer activity of Sir-SM2 against HeLa cervical cancer cells with MTT indicator revealed that the control cells showed cell morphology in normal conditions. However, the Sir-SM2 extract can inhibit the growth of HeLa cells from extract concentrations of 25–400 µg mL⁻¹ and successively result in an increasingly

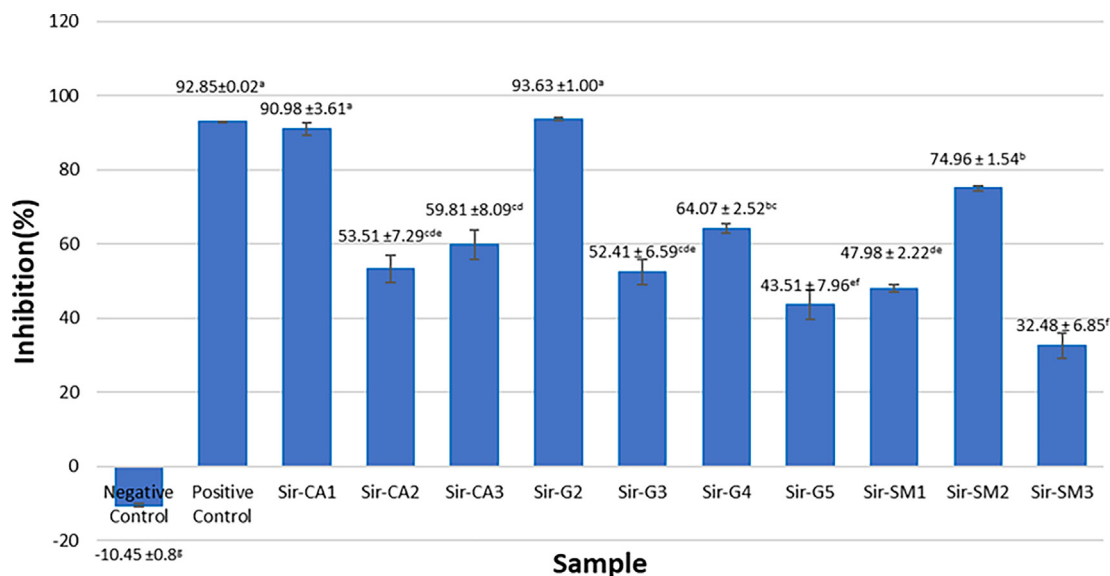


Fig. 1. Anticancer activity of endophytic fungi extract from soursop leaves against HeLa cells at 100 µg mL⁻¹ concentration.

Table 1

IC₅₀ value of ethyl acetate extract of soursop leaf endophytic fungi on HeLa cells.

Sample	IC ₅₀ (µg mL ⁻¹)
Extract Sir-CA1	8.47 ± 0.25 ^b
Extract Sir-CA3	155.34 ± 30.80 ^a
Extract Sir-G2	11.71 ± 1.05 ^b
Extract Sir-G4	36.48 ± 2.63 ^b
Extract Sir-SM2	29.14 ± 5.72 ^b

Different letters indicate significant differences in the Tukey test ($p < 0.05$)

damaged cell morphology (Fig. 2). Microscopic photos with 10x magnification of cervical cancer cells treated with Sir-SM2 endophytic fungi ethyl acetate extracts from 25 to 400 µg mL⁻¹ can be seen in Fig. 2.

Sir-SM2 has good cytotoxic activity against cervical cancer cells and the lowest cytotoxic activity against normal cells compared to other endophytic fungi ethyl acetate extracts, as reported by Arifni et al. (2017), and therefore selected as the best isolate.

3.2. Morphological identification of Sir-SM2 isolate

The selected endophytic fungi of Sir-SM2 isolate were observed on colony and spore, macroscopically and microscopically. Colonies grew well on MGYPA medium, reaching a 3.7–4.4 cm diameter within one week of incubation at 28 °C ± 2 °C. Colonies are concentric-round in shape with young hyphae white, greenish-blue, and dark green hyphae (asexual spores are green). The colony's surface is like cotton, smooth, and does not have aerial hyphae (hyphae that rise above the colony's surface).

Microscopic observation showed that Sir-SM2 isolate had somatic hyphae with septate character. Additionally, reproductive hyphae have parts, such as leg cells, erect conidiophores, metula, bottle-shaped conidiogens (phialides), chain conidium (leading to the genus *Penicillium*), round conidium (globose), hyaline color (transparent), and smooth conidium surface without ornament. Furthermore, the number of conidiogenous cells in one metula is a multiple of three.

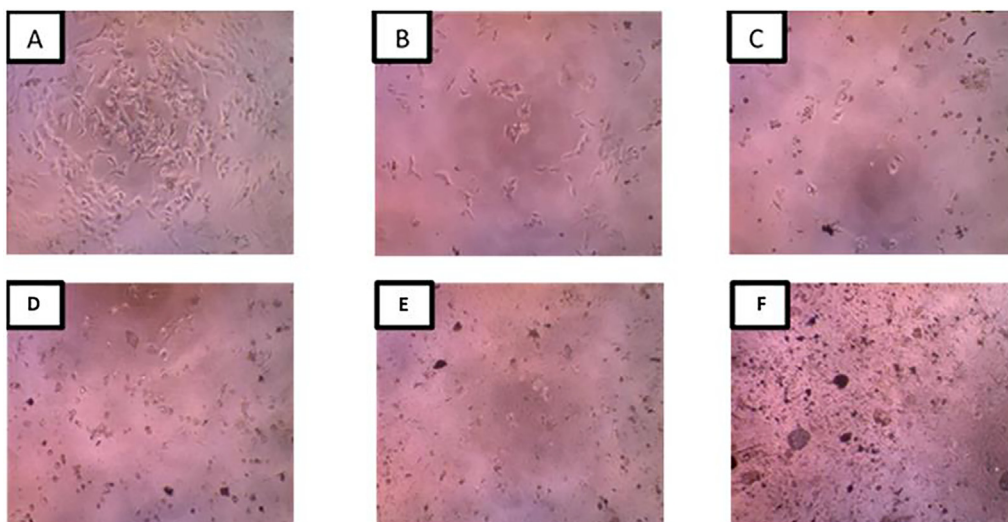


Fig. 2. Cervical cancer cells (HeLa cells) treated with soursop leaf endophytic fungi extract (Sir-SM2). Description: A = control without treatment, B = 25 µg mL⁻¹ concentration, C = 50 µg mL⁻¹ concentration, D = 100 µg mL⁻¹ concentration, E = 200 µg mL⁻¹ concentration, F = concentration 400 µg mL⁻¹ concentration (Canon inferted microscope, Dyno Eye camera, 1280 × 1024 unit: inch) at 10x magnification).

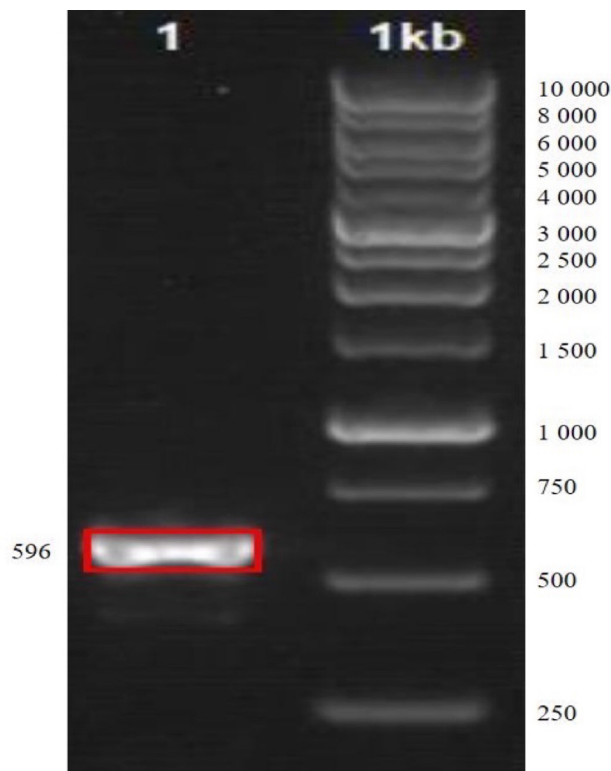


Fig. 3. PCR results of endophytic fungi isolate Sir-SM2.

3.3. Identification of Sir-SM2 isolate based on ITS Amplification.

Molecular-based identification was conducted to complete the information regarding the Sir-SM2 isolate. Based on the molecular approach, it showed a clear band in the 596 base pair region of DNA isolated from the endophytic fungi Sir-SM2 (marker 1 Kb). Additionally, the band produced from the PCR resulted in 1% agarose gel electrophoresis (Fig. 3).

Nucleotide sequence data alignment of the Sir-SM2 isolate was used to identify isolates by looking for the homology of the closest species sequence to the sequence data in the NCBI database/Gene Bank National Center for Biotechnology Information (Benson et al., 2005). The results showed a 99% similarity rate with *Penicillium crustosum* (*P. crustosum*). The phylogenetic tree analysis results reveal that the Sir-SM2 is highly similar to *P. crustosum* (Fig. 4). The phylogenetic tree was designed using a single ITS sequence with *Aspergillus fumigatus* and *Aspergillus niger* species as outgroups and 15 ITS sequences from the *Penicillium* strains of *P. crustosum*, *P. commune*, *P. expansum*, *P. sopii*, and *P. neocrassum* (*Lampiron jamessum* and *Peniensecil*). *Aspergillus* was chosen as the outgroup because it belongs to the group of imperfect fungi together with *Penicillium*. The stage of sexual reproduction in some species of *Penicillium* and *Aspergillus* has not been found (does not have a complete stage of sexual reproduction) (Nithiyaa et al., 2012).

4. Discussion

The statistical test results from the first screening showed that almost all samples had anticancer activity ($p < 0.05$) (Fig. 1). Five extracts, i.e., Sir-CA1, Sir-CA3, Sir-G2, Sir-G4, and Sir-SM2, were taken among the ten tested extracts based on the highest inhibition percentage. However, the five best extracts showed large variation in IC₅₀ values following the five concentration variation

testing, namely 25, 50, 100, 200, and 400 g mL⁻¹. Therefore, extracts from Cianjur, Garut, and Sukabumi with isolates names Sir-CA1, Sir-G2, and Sir-SM2, among the five best extracts, produced the highest IC₅₀ values and were chosen for further study. The selection was based on the criteria that a crude extract should be able to inhibit 50% of the cancer cells at a concentration below 30 μg mL⁻¹ (IC₅₀ < 30 g mL⁻¹) (Itharat et al., 2007).

The extracts of Sir-CA1, Sir-G2, and Sir-SM2 showed the highest anticancer activity with different values ($p < 0.05$) of 8.47, 11.71, and 29.14 μg mL⁻¹, respectively. Differences in values may be due to content differences of secondary metabolites in plants. The difference in metabolite compound contents from different areas may be attributed to differences in geological or environmental conditions. Temperature, water availability, solar energy, atmospheric quality, structure, air and soil composition, and reactions of soil and organisms will affect the content compounds in plants as hosts of these microbes form colonies (Nugraha et al., 2019).

Elisya and Murtini (2015) produced an IC₅₀ of 35.98 μg mL⁻¹ following testing of the soursop leaves ethyl acetate extract tablets on HeLa cells. At least 10 kg of soursop leaves is required to make tablet formulations with the best activity. Dewangga (2015) showed an IC₅₀ value of 77.096 μg mL⁻¹ against HeLa cells, using the chloroform-ethyl acetate fraction of soursop leaves. Chloroform extract from soursop leaves by Artanti et al. (2016) produced an IC₅₀ value of 127.3 μg mL⁻¹. Mardiyansih and Ismiyati (2014) showed that the IC₅₀ value or inhibition of cervical cancer cells (HeLa) was 10.016 ± 3.770 μg mL⁻¹. Suyatmi et al. (2012) found that soursop leaf extract inhibited the growth of 50% of HeLa cervical cancer cells at 97 μg mL⁻¹ concentration. All these researchers used soursop leaf extracts to test against HeLa cells with chloroform or ethyl acetate solvents, except Dewangga (2015), who used the fraction of soursop leaf extracts. Our study, which used endophytic fungi ethyl acetate extract, is different from these studies. Different materials and solvents may result in different extracted chemical compounds, affecting cytotoxicity. However, the cytotoxicity against HeLa cancer cells obtained in our study was better than Dewangga, 2015; Zuraida and Mariya, 2019. Furthermore, the research from Herlina et al. (2019) and Mardiyansih and Ismiyati (2014) produced a better IC₅₀ value compared to our study.

Soursop leaves have been traditionally used to treat cancer. The types and contents of biochemical compounds from soursop leaves grown in the natural condition are influenced by the environmental and plant growth conditions. Therefore, endophytic fungi isolated from soursop leaves and cultured on bioreactors under controlled conditions should maintain the continuity of chemical compound production with relative stability. Sir-SM2 fermentation using a bioreactor with controlled conditions showed that similar secondary metabolites were produced at both the laboratory and the factory scales and contained chemical compounds that act as anticancer (Hasan et al., 2020).

The extracts of Sir-SM2 was further tested using the MTT method for cytotoxic activity. The reduction by tetrazolium salts can reliably check proliferation. The basic of the MTT method is that the yellow tetrazolium salt is metabolically reduced by the active cells. This is partly caused by the dehydrogenase enzyme's action which reduces the equivalent amount of nicotinamide adenine dinucleotide (NAD) hydride and nicotinamide adenine dinucleotide phosphate (NADP). The resulting intracellular purple formazan can be dissolved and measured by spectrophotometer (American Type Culture Collection, 2017). The MTT can measure the rate of cell proliferation, and conversely, cell survival is reduced when a metabolic event causes apoptosis or necrosis. Furthermore, the MTT reagent produces a low absorbance value in the absence of living cells. Therefore, a linear relationship is found

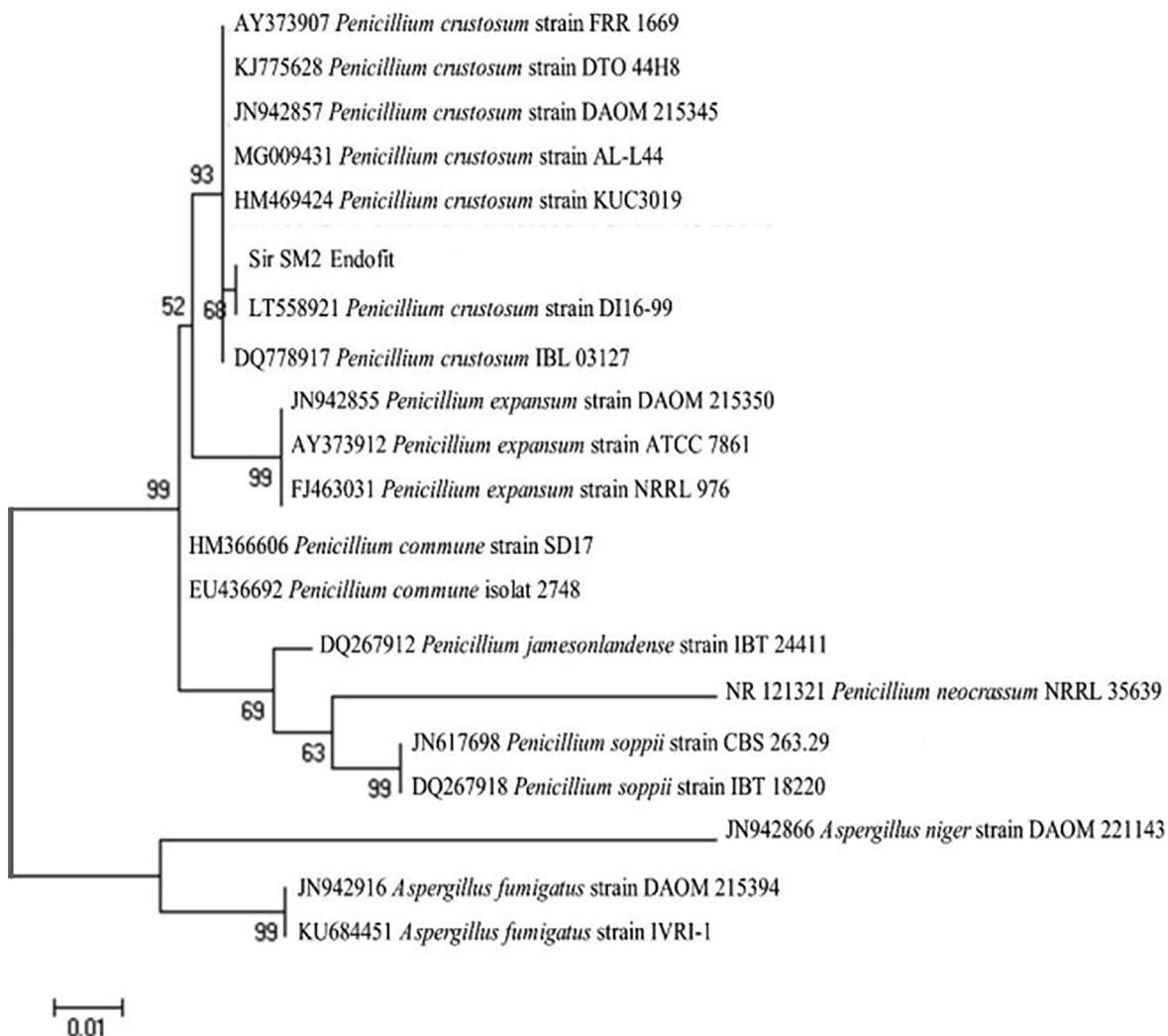


Fig. 4. Phylogenetic tree of endophytic fungi isolate Sir-SM2 with K2 + G model (Kimura, 2-parameter + gamma distributed).

between the number of living cells and the signal generated, allowing accurate quantification of changes in cell proliferation rates (American Type Culture Collection, 2017).

The MTT test result supported the IC_{50} data that the Sir-SM2 endophytic fungi extract can inhibit cancer cells. The HeLa cell inhibition mechanism can occur in three ways: the cell cycle arrest mechanism, cell cycle delay, and apoptosis. Apoptosis is the process of programmed cell death, a body regulation to get rid of unneeded or abnormal cells (Meng et al., 2020). Sir-SM2 endophytic fungi extract can induce cancer cell apoptosis, which may be caused by the metabolite compounds contained in the Sir-SM2 extract.

The Sir-SM2 isolate extract contained metabolite compounds that belong to the group of alkaloid i.e., (hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione, and hexahydro-3-[2-methyl propyl]-pyrrolo [1,2-*a*]pyrazine-1,4-dione) (Arifni et al., 2017). These compounds have alkylation ability, which can break DNA strands so that cells are damaged and cause cell death (Ser et al., 2015). The hexahydro-3-(2-methyl propyl)-Pyrrolo[1,2-*a*]pyrazine-1,4-dione compound can induce changes in cancer cell morphology due to DNA fragmentation, which indicates the ability of these alkaloid compounds to induce apoptosis in A549, and HeLa cancer cells (Lalitha et al., 2016). Hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione also has excellent antioxidant activity (Balakrishnan et al., 2015).

Antioxidants have been studied to trigger damage to the DNA double-strand of cancer cells, causing apoptosis (Meng et al., 2020).

The extracts of Sir-CA₁, Sir-G₂, and Sir-SM2 showed inhibition against HeLa cancer cells with IC_{50} values of $<30 \mu\text{g mL}^{-1}$. The IC_{50} data is quite interesting because endophytic fungi extract is more toxic to cancer cells than normal cells. Therefore, the endophytic fungi extract is supposedly safer with an IC_{50} of more than the limit set by Bray et al. (2018) (IC_{50} of $<30 \mu\text{g mL}^{-1}$).

These three extracts were tested against normal Chang cells to select the best candidate for their toxicity. An extract with the greatest IC_{50} value against Chang's normal cells will be chosen as the best extract. Sir-CA₁ and Sir-G₂ showed the best cancer inhibition values, but they were also toxic to Chang's normal cells (Arifni et al., 2017). The Sir-SM2 showed the lowest toxicity against Chang's normal cells at $63.69 \mu\text{g mL}^{-1}$, and its inhibition against HeLa cells was $29.14 \mu\text{g mL}^{-1}$. Therefore, Sir-SM2 is selected as the best isolate.

This study used doxorubicin as a positive control because of its wide use as chemotherapy in cancer treatment (Meredith and Dass, 2016). Doxorubicin at a lower concentration ($3 \mu\text{g/mL}$) in the study resulted in a large inhibition percentage against Chang's normal cells of 72.52%. Sir-SM2 extract can inhibit HeLa cell proliferation for $>50\%$ but damage normal cells at $<50\%$ at $25 \mu\text{g mL}^{-1}$

concentration. Therefore, the use of Sir-SM2 extract as an anticancer drug must be designed to specifically directly act on cancer cells for them to be less toxic to normal cells. Traditionally, patients with cancer are given a dose of cancer cytotoxic drugs in the trial phase to the maximum tolerated dose because it is assumed as the most effective dose (Thaker et al., 2017).

The best results from endophytic extract as an anticancer agent without damaging normal cells may be obtained through dosage, administration route, and treatment procedure modifications, which are important in research and further utilization. Chemical structure transformation and drug administration system application can reduce the toxicity of compounds from this extract, especially in normal cells. Additionally, isolation and use of selective compounds against cancer cells can be pharmacologically useful for further research.

Sir-SM2 was identified by observing several morphological characters, both macroscopically and microscopically. Macroscopic identification showed that the endophytic colony of Sir-SM2 was concentric spherical with young hyphae greenish-blue to white on the outside and dark green hyphae (asexual spores in green). Indrawati et al. (2021) reported that endophytes with dark green colonies with white surroundings on the PDA medium could be classified as *Penicillium* sp.

Microscopic identification showed that the endophytic fungi Sir-SM2 had rough cylindrical conidia, a characteristic specific to the genus *Penicillium* (Indrawati et al., 2021). Conidiophores singly arise from the mycelium or appear in synnemata, branching at the ends, penicillate, three-branched metula on one conidiophore, beam-shaped, and supporting three conidiogens on one metula. Conidiogen shaped like a bottle without any special ornaments (called phialid): conidia (phialospores) hyaline (transparent), conidia mostly globose to ovoid, in basipetal chains, and single-celled (no bulkhead in conidium). These microscopic characteristics point to the genus *Penicillium* sp. following the identification key of the genus (Nithiyaa et al., 2012).

Penicillium is one genus that can adapt to the most diverse environmental conditions (Nicoletti et al., 2014). *Penicillium* is commonly found in soil or as a contaminant in food, fruit, fiber, and other starchy materials. However, several studies have shown that *Penicillium* has evolved into endophytic fungi and has the potential as a new bioactive source and inhibit plants' stress tolerance (Yeshi et al., 2022). *Penicillium* is also known capable of synthesizing a large number of biochemical components and therefore has spurred numerous studies aimed at investigating its relationship with plant protection and opportunities for medicinal applications in humans, given the relevance of natural products for the development of new antibiotics, immunosuppressants, and medicines for anticancer (Nicoletti et al., 2014).

Fungal species identification at the DNA level uses Ribosomal DNA (rDNA) sequences in the ITS region. It has been used to establish phylogenetic relationships in many fungal groups (Nithiyaa et al., 2012). rDNA is the genomic coding region for the components of ribosomal RNA. The rDNA strands in eukaryotic organisms are located in the nucleus and mitochondria. Fungi are eukaryotic organisms. Conservative regions are found in fungal rDNA, namely the 18S, 5.8S, and 28S rRNA encoding genes, including the ITS region. The ITS area displays sufficient sequence variability to identify many fungal species down to the species level (Nilsson et al., 2008). DNA barcodes use standard sequences of 500–800 base pairs to identify species of the eukaryotic Kingdom using primers suitable for that taxonomic group (Yang et al., 2018).

The endophytic fungi isolate Sir-SM2 has a close relationship with *Penicillium crustosum* (*P. crustosum*) based on the phylogenetic tree (Fig. 4). The phylogenetic tree shows a strong clade formation in several species of *P. crustosum*, and Sir-SM2 belongs to that

clade. The confidence level is 93%; thus, Sir-SM2 can be classified as a species of *P. crustosum*. In the clade *P. crustosum*, the Sir-SM2 showed a 68% similarity with *P. crustosum* strain DI16-99. However, Sir-SM2 is possibly a new strain different from *P. crustosum* strain DI16-9 because this value is still below the standard bootstrap value (<80%). Therefore, further testing is needed by sequencing the gene regions other than ITS4 and ITS5, namely ITS1, ITS2, ITS3, tubulin, actin, small subunit, and large subunit, and other gene regions distinguish *P. crustosum* and Sir-SM2 in strain levels.

The score value, the sequence alignment value accuracy is an unknown nucleotide with the nucleotide sequence found in the GeneBank, was 1094. The higher the score obtained, the higher the level of homology of the two sequences. Query coverages, the percentage of nucleotide lengths consistent with the BLAST database, was 100%. Max identity, the highest value of percentage match between sequence query with an aligned database sequence, was 99%, and the E-value of 0 (zero) implies that the two sequences are indistinguishable (Pearson, 2013). The morphological identification showed that the soursop leaves endophytic fungi Sir-SM2 had similarities with the morphology of *P. crustosum* (Visagie et al., 2014).

P. crustosum is a cosmopolitan fungal species that can be isolated from meat, cheese, feed, vegetables, pomegranates, nuts, and seeds and is commonly found in rhizosphere soils from vegetable crops (Overy and Frisvad, 2003). *P. crustosum* is also found in the medicinal plant *Teucrium polium* L. (Hassan, 2017). *Penicillium* species are a widespread presence and are important due to the ability to synthesize mycotoxins and other active ingredients (Sonjak et al., 2005). In addition, *P. crustosum* synthesizes many volatile and non-volatile compounds (Overy and Frisvad, 2003).

5. Conclusion

Soursop leaves endophytic fungi ethyl acetate extracts are toxic to cervical cancer cells as indicated by inhibiting the proliferation of HeLa cancer cells *in vitro*. Crude ethyl acetate extract of isolated endophytic fungi Sir-SM2 has the potential as an anticancer. The Sir-SM2 shows high cytotoxicity to cervical cancer cells (HeLa cells) and produces the lowest toxicity on Chang's normal cells. Morphological identification suggested that the endophytic fungi Sir-SM2 belongs to the *Penicillium* genus. Furthermore, molecular identification using ITS shows that Sir-SM2 is similar to *P. crustosum*. The endophytic fungi Sir-SM2 is the potential for developing anticancer drugs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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