

ISOLATION AND ANTI-YEAST ACTIVITY OF SECONDARY METABOLITES OF SOURSOP LEAF ENDOPHYTIC FUNGI

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ABSTRACT

This study aimed to isolate the endophytic fungi of soursop leaves that have the potential as anti-yeast. Soursop leaves were collected from 3 locations in West Java, Indonesia. The secondary metabolites produced by the endophytic fungi were extracted with ethyl acetate and tested for anti-yeast activity against *Saccharomyces cerevisiae* and *Candida tropicalis*. Profile of extracts contents characterization was carried out by using thin-layer chromatography (TLC). Twelve monoculture soursop leaves endophytic fungi isolates from Sukabumi (3 isolates), Cianjur (3 isolates), and Garut (6 isolates). It showed different abilities to inhibit *C. tropicalis* and *S. cerevisiae*. The best extract is isolat from Cianjur (Sir-C1) and Sukabumi (Sir-S1). The stain patterns seen between the nine endophytic fungi extracts were similar. This indicates that the compounds in them may also be the same. But the extracts have a different absorbance for anti-yeast activities. This indicates that the total metabolites of each isolate are different. TLC results showed that ethyl acetate extraction produced almost the same metabolite components from nine endophytic fungi extracts, and all extracts had an anti-yeast activity with various absorbance. There are differences in the ability of 12 soursop leaf endophytic fungi to inhibit yeast model *C. tropicalis* and *S. cerevisiae*. Some extracts have similar TLC profiles, but their anti-yeast activity is not similar.

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1. INTRODUCTION

Soursop leaves are part of the soursop plant (*Annona muricata* L), widely used in traditional medicine to treat diseases like diabetes, asthma, and seizures (Nurrochmat et al., 2017). In Indonesia, soursop leaves use as herbal medicine to treat cancer by drinking a decoction of fresh soursop leaves. The fresh soursop leaves in boiled water can cause heat effects such as chemotherapy. Still, it only kills abnormal cells (cancer) and allows normal cells to continue to grow (Adri & Hersoelistyorini, 2013). Enweani et al. (2004); Audu et al. (2019) explained that soursop leaves contain flavonoids, tannins, alkaloids, saponins, calcium, phosphorus, carbohydrates, and vitamins (A, B, and C), phytosterols, calcium oxalate. Also, several other chemical constituents, including annonaceous acetogenins, can treat 12 types of cancer cells. Soursop leaves also have several therapeutic potentials such as antimicrobial (Solomon-Wisdom, 2014), wound healing (Paarakh et al., 2009), apoptosis-inducing (Moghadamtousi et al., 2014), chemo-beneficial (Soheil Zorofchian Moghadamtousi et al., 2015), antioxidant (Artini and Wahjuni, 2012) and other pharmacological compounds (Gajalakshmi et al., 2012).

Soursop plants are higher plants, and each higher plant has at least one endophytic microbe that lives in association and mutualism symbiosis with bacteria and fungi (Strobel & Daisy, 2003). Endophytic fungi are one of the most abundant endophytic microbes (Strobel & Daisy, 2003). Also, a rich source of bioactive secondary metabolites (Tan & Zou, 2001). Endophytic fungi are microorganisms found in plant tissue systems such as seeds, leaves, flowers, twigs, stems, and roots. Endophytic fungi can produce various functional compounds, including anti-cancer, antiviral, antibacterial, anti-yeast compounds, plant growth hormones, insecticides, and others (Strobel & Daisy, 2003). The research of (Hasan et al., 2020) found that soursop leaf extract had different activities against breast cancer cells.

The use of endophytic fungi is promising, so further research is needed to examine the benefits and activities of endophytic fungi, especially those found in soursop leaves. Utilizing endophytic fungi can be an innovative way to source bioactive compounds efficiently. Endophytic fungi isolated from medicinal plants such as soursop leaves will have the same or even better activity of bioactive compounds than their host plants because the mechanism of chemical changes by microorganisms is very similar to that which occurs in higher organisms. This is advantageous because the life cycle of endophytic fungi is shorter than that of the host plant and can be produced on a large scale using a fermentation process (Setyowati & Wardah, 2007).

S. cerevisiae can be used as a model of eukaryotic cells. The complete genome sequence is beneficial as a reference for humans and other eukaryotes (Engel et al., 2014). Ruta et al. (2020) explained that *S. cerevisiae* could be manipulated to be a perfect model as a eukaryotic cell for anti-cancer assays. Anti-cancer test using *S. cerevisiae* was based on the ability of the test compound to inhibit the growth of *S. cerevisiae* in the growth media. At the same time, *C. tropicalis* is a type of yeast that can be found in the wild and on the nose, throat, skin, vagina, and digestive tract in healthy individuals. However, many *C. tropicalis* attacks can infect patients either simultaneously or periodically (Lukisari & Harijanti, 2010).

This study aimed to isolate the endophytic fungi of soursop leaves from 3 locations in West Java, Indonesia, which are potentially anti-yeast. Also, compare the TLC profile of secondary metabolites of ethyl acetate extract from all endophytic fungi isolates of soursop leaves obtained. West Java is a center of soursop production in Indonesia, besides Central Java and East Java. Yeast *S. cerevisiae* and *C. tropicalis* were used to test the anti-yeast activity of crude ethyl acetate extract. The results of this study are expected to be used as a reference and consideration in further research on soursop leaf endophytic fungi extract as an anti-cancer.

2. METHODS

2.1 Materials

Soursop leaves were obtained from Sukabumi, Cianjur, and Garut, West Java, Indonesia. Yeast cultures were obtained from INACC (Indonesian Culture Collection).

2.2 Endophytic Fungi Isolation

Potato Dextrose Agar (PDA) media was used to grow the fungi, added with amoxicillin. PDA was prepared by adding 24 g Potato Dextrose Broth (PDB, Himedia) and 3 g, solved in 1 L aqua dest. Soursop leaves were washed under running water, then soaked for 10 minutes, put in laminar, then cut into squares of about 1 cm x 1 cm, cut into five pieces of leaves. The sample pieces were then put into a petri dish to gradually sterilize the samples' surfaces using an antiseptic solution. First, samples were soaked in 75% ethanol for 1 minute, then sterilized with NaOCl for 5 minutes, and 75% ethanol for 0.5 minutes, then washed with sterile distilled water. The sample pieces were placed on sterile filter paper and air-dried at room temperature for 2 hours. The dried sample pieces are then transferred to culture media (PDA + Anti-Bacterial / amoxicillin). Samples were incubated at room temperature for 3-7 days until the fungus grew. Selected colonies that grow on the sample pieces grown on new media for further isolation. Isolates were then incubated at room temperature for 24 hours. When macroscopically different colony growth was found in the same petri dish, then they were separated until pure isolate was obtained.

2.3 Isolate Cultivation (Agusta, 2013)

The pure isolates were grown in 200 mL of Yeast Malt Broth (YMB, Himedia) and incubated at 27°C, shaken at 120 rpm for 21 days.

2.4 Fungi Culture Extraction

Fungal metabolites were then extracted to obtain the main compound of endophytic fungi. Cultures broth of 200 ml was then added with 300 ml of ethyl acetate solvent in Erlenmeyer 500 ml, shaken manually for 15 minutes. This was done to dissolve the endophytic fungi compounds obtained in the ethyl acetate solution. Then the fraction solution is poured into the boiling flask; the second layer of the fraction should not be included in the boiling flask. The top layer of the fraction was evaporated with an evaporator under a vacuum; the water bath temperature was 30 °C. This process is carried out until a small amount of solution remains, then freeze-dried and weighed.

2.5. Thin Layer Chromatography Analysis

The endophytic fungi extract was then analyzed by thin-layer chromatography to compare the spots among the plates dripping with different extract solutions to illustrate the compounds contained in it. The extract was dissolved in acetone with a concentration of 5 mg/mL; 10 μ l of each extract was spotted on the available TLC plates from the same starting end. The developer vessel or chamber is filled with Hexane: Ethyl Acetate eluent with various ratios to get the best results. After the plate was spotted with each fungi extract, it was inserted into a chamber that already contained eluent, then eluted until the solvent limit or the front line approached the end. The tip was marked with a line using a pencil. The plate is removed from the chamber and dried using a hairdryer when finished.

The faint spots formed on the plate were then observed under UV light at 254 and 366 nm. Certain spots will Fluorescence. Detection is then carried out using a color-forming reagent, which is sprayed evenly on the surface of the plate. The reagents used include Vanillin or Serum. The plate that has been colored evenly is then heated in a bath, until a clear color appears on the spots.

2.2.7 Anti Yeast Test

The anti-yeast activity was tested by the paper disk method, according to the guidelines of the National Committee for Clinical Laboratory Standards Institute (CLSI 2006) and Sung & Lee (2007). Yeast of *C tropicalis* and *S. cerevisiae* was grown on NA media were taken as much as two oses and then cultivated in YMB media at 37 °C for 48 hours with a shaking incubator (100 rpm).

Yeast cell suspension was diluted 10x by adding YMB media. Diluted yeast cells suspension of 100 μ L then poured into a petri dish containing YMA media and leveled so that it is homogeneous. A blank Antimicrobial Susceptibility disc was dipped in the test extract solution, then placed on top of the test yeast inoculant. Acetone was used as a solvent and negative control. The positive control was Nystatin (anti-yeast), each with a 5 mg/ml concentration. Inoculants that have been given stock solution are incubated at room temperature for 68 hours, then the inhibition zone is observed.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1 Endophytic Fungi Isolation

Isolation of the endophytic fungus using three different types of soursop leaves. Namely soursop leaves from Sukabumi, Cianjur, and Garut. After being incubated on PDA media for 3 days, the endophytic fungi that emerged from soursop leaves were purified to obtain pure monoculture isolates. From soursop leaves (*Annona muricata* L). A total of 12 isolates of endophytic fungi were obtained, which are presented in Figure 1. The twelve isolates of this endophytic fungi consisted of 3 isolates from soursop leaves from Sukabumi, three isolates from Cianjur, and six from Garut. These isolates were observed macroscopically for their colonies, including colony color, texture, edge, and diameter size (Figure 2).

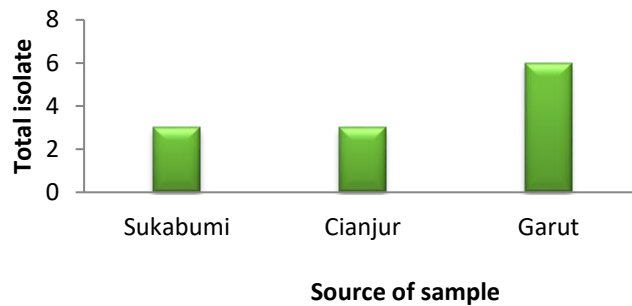


Figure 1 Number of endophytic fungi isolates from three regions.



Figure 2 Soursop leaf endophytic fungi isolated from Sukabumi (left), Cianjur (middle), and Garut (right)

3.1.2 Isolate Cultivation

Endophytic fungi are culturable (can be grown under artificial conditions) (Agusta, 2013). Endophytic fungi cultivation media contains carbon, nitrogen, sulfur and phosphorus, metallic minerals, vitamins, and of course, water (Backer et al., 2018). In this research, the media type is YMB. Cultivation was carried out until the culture isolates were about 21 days (Figure 3).



Figure 3 Cultivation results of soursop leaf endophytic fungi isolate in YMB media.

In this study, the production medium was maintained at room temperature and cultivated on the production medium for 21 days. Of the twelve fungi that were cultivated, each had a different shape and texture of the appearance of the fungi culture; this shows that the twelve endophytic fungi that were obtained were each other. In addition to the cultivation results, there was only one culture formed in the media; this also indicates that the cultivated fungi isolates were monocultures.

3.1.3 Endophytic Fungi Culture Extract

Endophytic fungi extract (Table 1) is the extract obtained after the extraction process using ethyl acetate solvent, then evaporated with an evaporator and dried with the addition of nitrogen. The extract is then stored in the refrigerator for later use in the next test.

Table 1 Weight of extracts obtained from twelve endophytic fungi cultures

Sample No.	Sample Code	Weight of Ethyl Acetate extracts (g)
1	Sir-S ₁	0.0050
2	Sir-S ₂	0.0046
3	Sir-S ₃	0.0072
4	Sir-C ₁	0.0022
5	Sir-C ₂	0.0028
6	Sir-C ₃	0.0128
7	Sir-G ₁	0.0069
8	Sir-G ₂	0.0115
9	Sir-G ₃	0.0064
10	Sir-G ₄	0.0095
11	Sir-G ₅	0.0093
12	Sir-G ₆	0.0441

3.1.4. Thin Layer Chromatography Analysis Results

Soursop leaf endophytic fungi extract was analyzed by thin-layer chromatography to see the pattern of compounds from soursop leaf. Endophytic fungi extract compared to soursop leaf extract. A similar way indicates the possible similarity of compounds in the extract between the endophytic fungi extract and the leaf extract. Previously, an eluent comparison trial was conducted, namely, Hexane: Ethyl acetate (8:1), (6:1), (4:1), (2:1), so that the appropriate eluent could be determined (Figures 4 and 5).

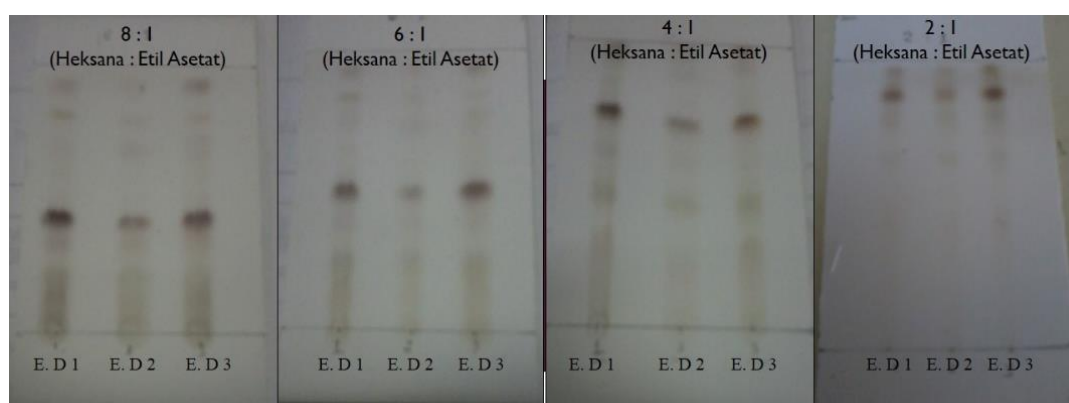


Figure 4 TLC profile (Thin Layer Chromatography) soursop leaf extract (ED) with three types, E.D1 (Soursop Leaf Extract Sukabumi Asam), E.D2 (Soursop Leaf Extract Sweet Sukabumi), E.D3 (Soursop Leaf Extract Cianjur acid) with eluent hexane: ethyl acetate using various ratio. The dye used is cerium.

Based on the results of the fluent ratio, the profile of the fungi extract was tested with hexane, and ethyl acetate as a solvent in a ratio of 2:1.

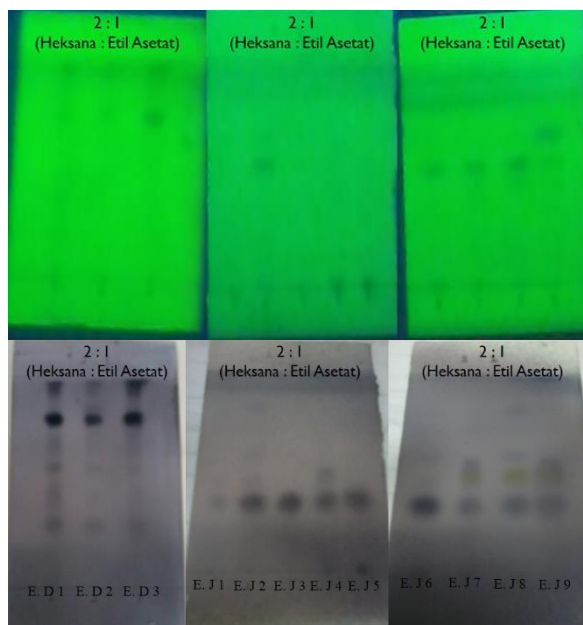


Figure 5 TLC profile (Thin Layer Chromatography) soursop leaf extract (E.D) and endophytic fungi extract (E.J) with vanillin dye and UV 254nm.

3.1.5 Anti-yeast Activity Screening with MTT Indicator

Based on the extraction results in Table 1, the yeast was tested as part of the anti-cancer model testing using *C. tropicalis* and *S. cerevisiae*. The test results are shown in Figures 6 and 7.

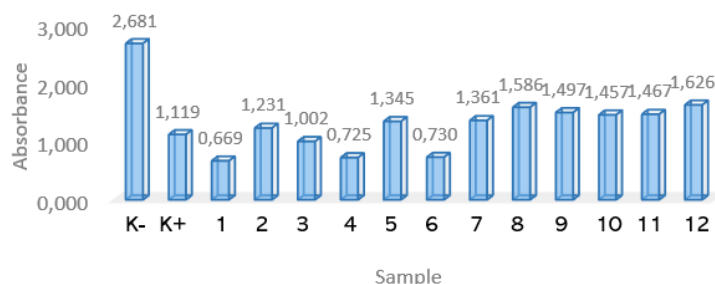


Figure 6 Screening of yeast activity (*C. tropicalis*) endophytic fungi extract. Sample numbers 1 to 12 correspond to the sample numbers in Table 1

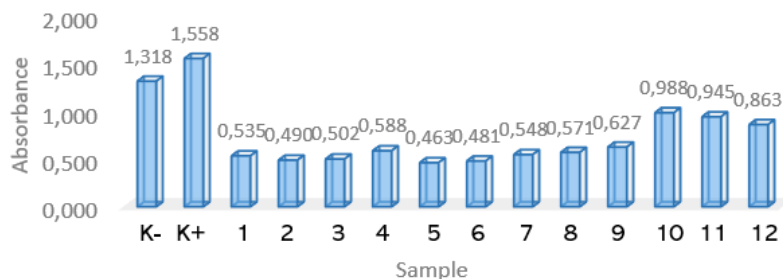


Figure 7 Screening of yeast activity (*S. cerevisiae*) endophytic fungi extract. Sample numbers 1 to 12 correspond to the sample numbers in Table 1

3.2. Discussion

Endophytic microbes live in plant tissues for a certain period and can live by forming colonies in plant tissues without harming their hosts (Marag & Suman, 2018). From each soursop leaf, endophytic fungi grow with different morphology. This indicated that colonies are different species. Thus, there will be other secondary metabolites and functions.

The selection of eluents was based on several conditions (Polak & Pajurek, 2021), namely providing an R_f value between 0.2-0.8 and providing a fairly good selectivity for the components of the active substance to be separated. The simplest system is a mixture of two organic solvents because it has optimal elution power (Parris, 1976).

TLC plates that have been eluted and sprayed with cerium or vanillin sulfuric acid stains show different stains in each hexane: ethyl acetate ratio. Hexane is nonpolar, while ethyl acetate has a polarity of 4.4, so the eluent ratio of hexane: ethyl acetate 2:1 is more polar. The TLC profile shown in Figure 3 shows that the ratio of hexane: ethyl acetate 2:1 has the best-separated spots. Thus, the eluent used in the TLC extract of the soursop leaf endophytic fungus was hexane: ethyl acetate with a ratio of 2:1.

The selection of the right eluent ratio was then used for TLC analysis on the soursop leaf endophytic fungi extract. Soursop leaf endophytic fungi extract, which was previously dry, was added with acetone to dissolve it again. The addition of acetone was adjusted to the dry weight of the endophytic fungi extract; the desired final concentration from the addition of acetone was 5 mg/ml. After being dissolved in acetone, the nine extracts were dripped onto the TLC plate and stained with vanillin sulfuric acid.

The appearance of the stains formed on the endophytic fungi extract TLC plate after staining with vanillin sulfuric acid was then compared with the stains created on the leaf extract TLC plate. Previously, the TLC plate was also exposed to UV light at 254 nm to see the pattern of stains formed and visible. The comparison of the patterns formed shows that the pattern of spread of stains on the plate between soursop leaf extract and endophytic fungi extract looks different (Figure 4). It's just that the appearance of stain lines that are similar to each other but not too conspicuous, so there may be the same compound content but with different concentrations.

In the TLC analysis, it was seen that there was a pattern of stains formed on staining with vanillin, but it was not visible when UV irradiation was carried out at 254 nm. This is because the staining pattern in UV is not very clear, so stained with vanillin shows a clear stain pattern. The stain patterns seen between the nine endophytic fungi extracts were similar; this indicates that their compounds may also be the same.

The results of screening for anti-yeast activity (Figure 6-7) were carried out to determine which extract had the best anti-yeast activity by using the indicator of MTT reduction absorbance value. In the percentage of *C. tropicalis* anti-yeast activity screening results, it is known that extract one and extract 4 have the smallest absorbance value (Figure 6), where a small absorbance value indicates the low viability of yeast cells. The smaller the absorbance value obtained, the better the extract as an anti-yeast. So extract 1 (Sir-S₁) and extract 4 (Sir-C₁) were chosen as the best extracts to be tested for MTT reduction with various concentrations. TLC profile of extracts 2 and 3 is like that of extract 1, indicating similar content of some metabolites. However, activity against *C. tropicalis* of extracts 2 and 3 is not similar to that of extract 1. This data indicates that even when a crude extract contains some same metabolites as an active extract, it does not mean that the extract is active against *C. tropicalis*. Meanwhile, in the percentage of screening results for the anti-yeast activity of *S. cerevisiae*, it was known that extract five and extract 6 had the smallest absorbance values. However, in the screening test with the yeast *S. cerevisiae*, the best extracts were extracted five and extract 8; this was because the purple color of extract 6 was greater than that of extract 8, where the appearance of purple color in MTT indicated dominant yeast cell activity (Figure

7).). In a study conducted by Minarni et al. (2017), there are endophytic fungi of soursop leaves that have potential as anti-cancer breasts, while research conducted by (Arifni et al., 2017) found potential as an anti-cancer of the colon and cervix. The three potentials of soursop leaf endophytic fungi, which were carried out as different anti-cancer agents were obtained from other types of fungi.

4. CONCLUSION

We obtained 12 endophytic fungi isolates from soursop leaves consisting of 3 isolates from Sukabumi, three isolates from Cianjur, and six isolates from Garut. All isolates can be extracted with ethyl acetate and showed anti-yeast potential against *C. tropicalis* and *S. cerevisiae*. Some extracts with similar TLC profiles showed that extraction using ethyl acetate produced almost the same metabolite components that might play a role as anti-yeast. There are differences in the ability of 12 soursop leaf endophytic fungi to inhibit yeast model *C. tropicalis* and *S. cerevisiae*. Some extracts have similar TLC profiles, but their anti-yeast activity is not similar.

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