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Anticancer activity test of ethyl acetate extract of endophytic fungi isolated from soursop leaf (*Annona muricata* L.)

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ABSTRACT

Objective: To analyze anticancer activity of an ethyl acetate extract of endophytic fungi isolated from soursop leaf (*Annona muricata* L.).
Methods: Anticancer activity of fungal extracts was determined by observing its toxicity against MCF-7 (Michigan Cancer Foundation-7) cells *in vitro* by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method. At an extract concentration of 100 μg/mL, 4 isolates out of 12 showed high activity against the cancer cell growth. The four isolates were then selected for further IC_{50} determination, by measuring the inhibition of cancer cell proliferation at extract concentration of 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL and 400 μg/mL.
Results: Results showed that isolate Sir-G5 had the highest anticancer activity with an IC_{50} of 19.20 μg/mL. The best isolates were screened again using a normal cell (Chang cells) to determine its toxicity against normal cells. Results indicated that the extracts do not affect the proliferation of normal cells. Molecular identification showed that the fungal isolate Sir-G5 has a close relationship with *Phomopsis* sp.
Conclusions: The endophytic fungi isolated from soursop leaf has the potential to be used as a source of anticancer agents.

1. Introduction

Cancer is one of leading causes of death in the world. It was reported [1] the total world civilian death from cancer is about 7.6 millions and this number is predicted to increase to 13.1 millions in 2030 [2]. Globally, breast cancer is the most aggressive cancer in woman with an estimated 1.38 million new cases per year [3].

Combinations of some treatments such as chemotherapy, operative, radiotherapy, immunotherapy and photodynamics therapy are commonly used to treat cancer [4]. However, these can induce dangerous side effects if the cancer treatments affect normal cell metabolism [5]. In addition, cancer resistance could exist as a result of anti-apoptotic of drugs used in therapy [6]. Herbal plant treatments can therefore be used as an alternative medicine potentially without side effects, and do not inhibit the anti-apoptotic pathway at low doses [7].

One plant that has been used as an anticancer agent is *Annona muricata* (*A. muricata*), especially its leaves. *A. muricata* is a member of the Annonaceae family, also known as soursop. It is an evergreen plant mostly distributed in tropical and subtropical regions of the world. Many studies have reported that extracts of *A. muricata* have anticancer activity [8]. It was shown that soursop leaves have great potency as anticancer agents because of their ability in inhibiting cancer cell growth [9]. Cancer treatment by soursop leaves is also more promising and safer compared to chemotherapy or radiation [10]. Furthermore, soursop leaf extracts are used in cancer cell recovery and show inhibition of cancer cell growth after being tested in mice induced with a carcinogen [11].

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Dicotyledonous plants such as soursop are predicted to have endophytic microbes which are a potential medicinal sources. Endophytic microbes usually create symbiotic interactions with plant tissues. A number of plant endophytic fungi have been shown to have anticancer activity [12,13]. Endophytic microbes used in this research were limited to endophytic fungi based on their morphology. The present study was intended to test the anticancer activity *in vitro* of ethyl acetate extracts of soursop leaf endophytic fungi in the search for anti-breast cancer agents.

2. Materials and methods

2.1. Extraction of soursop leaf endophytic fungi

Soursop leaf endophytic fungi isolates were cultivated and then extracted to obtain the main compounds they harbor. Cultures of endophytic fungi isolates in 200 mL YMB media were combined with 300 mL of ethyl acetate, the mixture was then shaken manually for 15 min. The top fraction of the mixture was then cleanly poured into a flask. This fraction was concentrated using a rotary vacuum evaporator at 40 °C, and then dried with nitrogen. The quantity of the extract was determined by weight [14].

2.2. Cytotoxic activity test

Extract of soursop leaf endophytic fungi was dissolved in DMSO as 10% (w/v) stock solution. Working solution (1% v/v) was made by dissolving the extract in RPMI-1640 (Roswell Park Memorial Institute) medium. Extracts from 12 fungal isolates were subjected to anticancer activity test using MCF-7 cancer cell line at extract concentration of 100 µg/mL. Four extracts with highest activity based on IC_{50} value were then retested on MCF-7 cells at a range of concentrations: 25, 50, 100, 200 and 400 µg/mL. The most active extract was selected and tested with Chang normal cells at the same concentration. DMSO was used as a negative control while doxorubicin was used as a positive control. As much as 20 µL of each solution was respectively added to a microplate which was then filled with 100 µL of sustainable cancer cells (7.5×10^4 cells/mL). The mixture was incubated for 24 h at 37 °C in 5% CO_2 incubator. Surviving cells were determined by counting with an ELISA reader at 595 nm. Absorbance data from the ELISA reading were converted into % cell inhibition according to the equation:

$$\text{Cell inhibition} = \{(\text{Abs. value of control} - \text{Abs. value of sample}) / \text{Abs value of control}\} \times 100\%$$

The correlation of cell death and extract concentration was then analyzed by using a line regression test. The extract concentration which resulted in 50% of cancer cell growth (IC_{50}) inhibition was then calculated [15,16].

2.3. Molecular identification of endophytic fungi based on Internal Transcribed Spacer (ITS) gene

Identification of fungi isolate was carried out using molecular based analysis of Internal Transcribed Spacer (ITS) locus on the ribosomal RNA gene. DNA isolation was initiated by growing fungal isolates in liquid Potato Dextrose Broth (PDB) medium followed by incubation for 72 h. Mycelia biomass was harvested for DNA extraction. Fungal DNA extraction was done using nucleon PHYTO pure reagent (Amersham LIFE SCIENCE). PCR amplification of ITS DNA fragment was conducted using the ITS 4 primer (reverse): 5′-TCC TCC GCT TAT TGA TAT GC–3′ and ITS 5 primer (forward): 5′-GGA AGT AAA AGT CGT AAC AAG G–3′ [17,18]. PCR product was analyzed by agarose gel electrophoresis according to [19].

PCR product purification was done with PEG precipitation method [20] and followed by PCR sequencing cycles. DNA sequencing was carried out using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). The DNA sequence obtained was used to construct phylogenetic tree using MEGA 5 [21,22].

2.4. Data analysis

Research design used was based on a Factorial, Completely Randomized Design. The factors were extract sources (Sir-G5, Sir-SM2, Sir-G6 and Sir-G2) and extract concentrations. Data were obtained from analyzing the variance of measured variables (two-way ANOVA). If this showed significant differences, then analysis was continued to Duncan test with α 0.05. Statistical tests were carried out using MS Excel 2013, SAS 1.9.3 and Minitab 15 [23].

3. Results

3.1. Cytotoxic activity of extract of leaf endophytic fungi

Extracts generated from soursop leaf endophytic fungi and their cytotoxic activity are presented in Table 1. Extracts varied in quantity, indicating yield variation. Extracts were refrigerated until used. Results of cytotoxic assays showed that 12 extracts of endophytic fungi inhibited MCF-7 cancer cell proliferation. The extracts with highest activity were Sir-SM2, Sir-G6, Sir-G2 and Sir-G5.

The four extracts with highest activity were then retested to determine the most active extract. Their anticancer activities are presented in Figure 1.

Table 1
Amounts of extract obtained from soursop leaf endophytic fungi and their anticancer activity on cancer cell MCF-7 at 100 µg/mL.

No	Sample	Ethyl-acetate extract (mg dry weight)	% Cell inhibition
1	Sir-SM2	30	31.98
2	Sir-G3	10	24.72
3	Sir-CA_1	20	19.88
4	Sir-G6	30	26.71
5	Sir-SM1	30	16.94
6	Sir-G2	40	25.50
7	Sir-G5	20	79.34
8	Sir-CA2	20	20.14
9	Sir-G4	30	17.55
10	Sir-G1	20	20.22
11	Sir-SM3	20	0.95
12	Sir-CA3	20	0.00

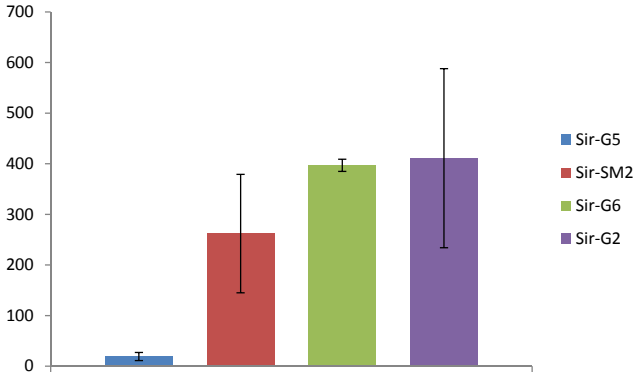

Figure 1. IC_{50} value of ethyl acetate extract of soursop leaf endophytic fungi.
Bar (I) represents standard deviation.

The most active extract (Sir-G5) with lowest IC_{50} value was retested with MCF-7 breast-cancer cells to determine its effects on MCF-7 breast-cancer cells. Cells used were living cells, counted with a hemacytometer, which differentiated between living cells and dead cells by tripan blue coloration. Living cells were spheroidal and pellucid in the middle, while dead cells have an irregular shape and blue color, and devoid of nucleus. Results are shown in Figure 2. Control cells appear normal morphology. Treatment with extract of soursop leaf endophytic fungi at various concentration affected the morphology of the MCF-7 cancer cells compared to the negative control.

The most active extract (Sir-G5) was retested with normal Chang cells. Anticancer agent can be expected to have selective toxicity, in that they inhibit cancer cells without affecting the normal cells [24]. Data showed that extract of soursop leaf endophytic fungi were not toxic to Chang normal cells indicated by the high IC_{50} value (1 258.92 μg/mL). Figure 3 shows that Chang normal cells have a normal proliferation phase, fixes morphologically and fills the whole well.

3.2. Endophytic fungi identification based on nucleotide sequence of rRNA Internal-Transcribed-Spacer (ITS)-gene

Genomic DNA isolation of Sir-G5 soursop leaf endophytic fungi was carried out using nucleon PHYTO pure reagent (Amersham LIFE SCIENCE). The isolated genomic DNA was then used as a template for PCR amplification of rRNA ITS gene. The rRNA ITS gene fragment (550 bp) was successfully amplified using the ITS 4 and ITS 5 primers. DNA fragment of the same size was also obtained by [25] who amplified the corresponding DNA region from endophytic fungi of plant genus Annona.

Phylogenetic tree analysis (Figure 4) based on the rRNA ITS gene fragment showed that the endophytic fungi isolated from the soursop leaf has a close genetic relationship with *Phomopsis* sp. indicated by their close genetic distance. Sequence of the rRNA ITS gene indicated that the isolate has high genetic relationship with *Phomopsis* sp. LH243 with bootstrap value of 97%, and homology of 99.998% and similarity index of 99%.

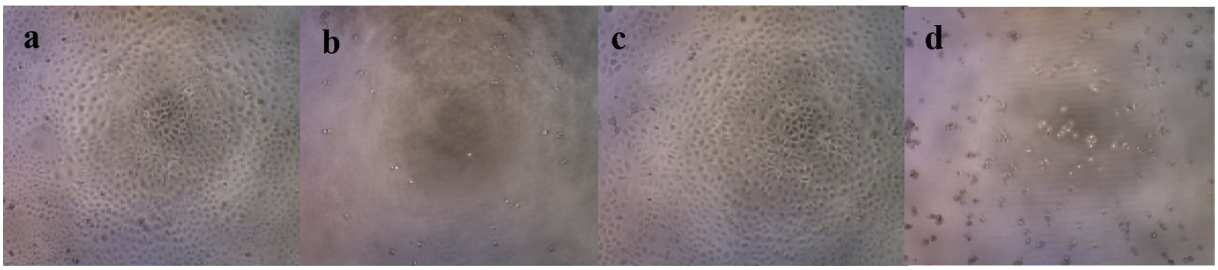

Figure 2. MCF-7 breast-cancer cells treated with extract of soursop leaf endophytic fungi (Sir-G5).
Description: a = control, MCF-7 breast-cancer cells without treatment, b = MCF-7 breast-cancer cells treated with extract at concentration of 400 μg/mL, c = MCF-7 breast-cancer cells treated with extract at concentration of 25 μg/mL, and d = MCF-7 breast-cancer cells treated with doxorubicin of 0.5 μg/mL (Canon Inverted Microscope, Dino Eye Camera with the size of 1 280 × 1 024 unit: inch) 10× magnification.

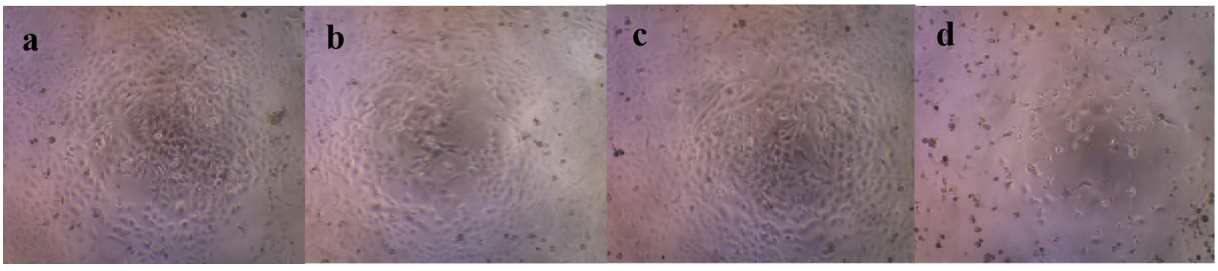

Figure 3. Chang normal cells treated with extract of soursop leaf endophytic fungi (Sir-G5).
Description: a = control, Chang cells without treatment, b = Chang cells treated with extract at concentration of 400 μg/mL, c = Chang cells treated with extract at concentration of 25 μg/mL, d = Chang cells treated with doxorubicin concentration of 0.5 μg/mL (Canon Inverted Microscope, Dino Eye Camera with the size of 1 280 × 1 024 unit: inch) 10× magnification.

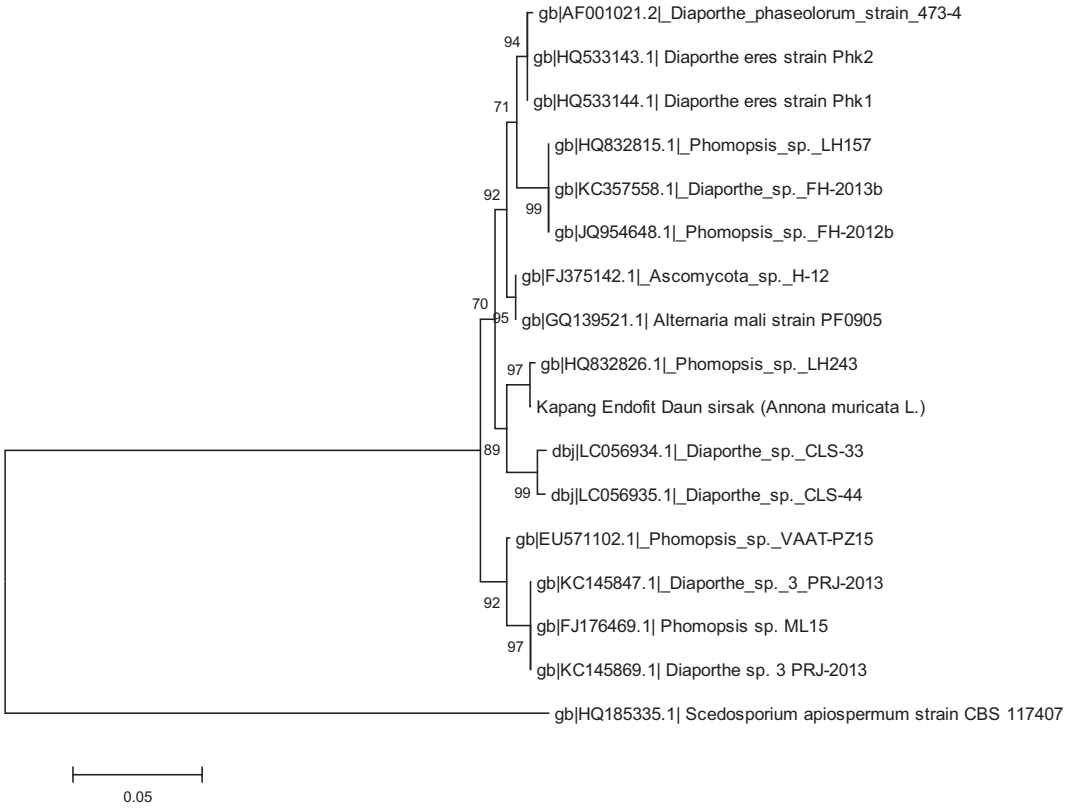

Figure 4. Phylogenetic tree of endophytic fungi of soursop-leaf (*Annona muricata* L.).

4. Discussion

There is a constant need to develop novel, effective, and affordable anticancer drugs. In addition to *in vitro* and *in vivo* methods [26,27], *in silico* approach has also been used to predict molecules with anticancer activity [28,29]. Here we report *in vitro* study on anticancer activity of ethyl acetate extract of endophytic fungi isolated from soursop leaf (*A. muricata* L.). Extracts of the endophytic fungi are expected to contain compounds with anticancer activity. The choice of appropriate solvent could improve extraction efficiency. Solvent used in this research was ethyl acetate. Ethyl acetate is a solvent of choice for extraction because it is easy to evaporate, is non-hygroscopic and has weak toxicity. Ethyl acetate tends to be semipolar allowing it to attract active compounds in soursop leaf endophytes. Other researchers have successfully used ethyl acetate to extract active compounds from endophytic fungi [30].

The cytotoxic activity of the endophytic fungi extract has been determined based on its effective concentration (IC_{50}) to inhibit proliferation of breast cancer cells [31]. An *in vitro* cytotoxicity test was selected for initial screening of extracts with anticancer potential. The MTT colorimetric assay has been widely used to test cytotoxic activity *in vitro* [32]. The MTT assay is accurate because of its ability to describe the relationship between the amount of active cells with absorbance obtained from measuring its 50% inhibition concentration value (IC_{50}) [33]. The less the IC_{50} value, the higher the potential of the tested extract to inhibit cell proliferation. The principle of the MTT assay is to measure the activity of mitochondrial dehydrogenase in converting MTT into formazan. The concentration of formazan, which has a blue color, can be determined with a visible spectrophotometer and it has positive correlation with the number of living cells because the reduction event only exists when the mitochondrial reductase is produced (mitochondria is still active and this indicates that the cell is alive) [34].

Extracts of samples collected from 12 different locations showed activity in every test. Four extracts (Sir-G5, Sir-SM2, Sir-G6 and Sir-G2) showed significant anticancer activity. The lower the IC_{50} value the higher the anticancer activity of the extract. Extract from the fungal isolate Sir-G5 (from Garut) showed the highest anticancer activity based on average value of IC_{50}. Activity could be affected by the local habitate and location of the soursop that may lead to different types and amounts of bioactive compounds in each endophytic fungi isolate. It was reported [35] plant secondary metabolites will vary according to location.

This research employed number of cancer-cell culture suspension at 7.75×10^6 cells/mL. Cancer cells of that amount was expected to survive for 24–28 h. Determination of 24–48 h incubation was intended to prevent the lack of available nutrition during cell growth. In the preparation of samples, endophytic-fungi-extract of soursop leaf was dissolved in DMSO. Dilution of the main sample solution in DMSO was made using RPMI medium. RPMI medium is one of the popular media for culturing cancer cells [36].

Cellular morphological change could be caused by apoptosis. The mechanism of apoptosis usually used to describe anticancer mechanism brought about by various drug compounds. The MCF-7 cell growth was successfully inhibited by soursop leaf endophytic fungi extract. This could be caused by apoptosis mechanism leading to cell morphological change. Cellular morphological change associated with apoptosis involves steps: cell density decreasing, chromatin condensation and fragmentation, and

nucleus fragmentation [37]. Extracts of soursop-leaf-endophytic fungi may protect normal cells from becoming cancerous by binding to mitochondrial proteins of cancer cells to induce apoptosis without breaking neighbouring cells [38].

Photographic evidence showed different morphology between cells treated with extracts at concentrations of 25 μg/mL or 400 μg/mL and cells treated with doxorubicin at concentration of 0.5 μg/mL. MCF-7 cancer cells treated with extract at a concentration of 25 μg/mL showed significant proliferation inhibition, in line with results of [8] that used soursop leaf extracts as an anticancer agent. Similarly, soursop leaf extract was found to have low IC_{50} value [39].

It was found that ethyl acetate extracts of *Eupatorium odoratum* has potential cytotoxic activity to MCF-7 cell by increasing LC3-A protein expression, and inducing cell death [40]. The American National Cancer Institute defined an extract with strong cytotoxic activity as an anticancer agent if it shows an IC_{50} value of less than 30 μg/mL [10]. Data from our MTT assay showed that ethyl acetate extracts of soursop leaf endophytic fungi has an IC_{50} value of 19.20 μg/mL. Therefore, our ethyl acetate extracts can be categorized to have strong cytotoxic activity.

Cytotoxic activity of the fungal extract to MCF-7 cells could be caused by apoptosis induction in the cells. Apoptosis is programmed cell death on physiological and pathological condition to avoid the other cells rupturing [41]. The capacity of the extract to induce apoptosis could be due to the presence of secondary metabolites naturally produced by the endophytic fungi. Cell viability analysis and MTT tests showed that extracts of endophytic fungi isolated from soursop leaf might induce apoptosis in MCF-7 cells by an intrinsic pathway related to cytochrome *c* migration to the cytosol and the dissociation of the mitochondrial membrane [41,42]. An apoptotic intrinsic pathway is initiated by gene expression of the Bax protein. This protein interacts with mitochondria to release cytochrome *c* into the cytosol and at the same time increases Ca^{2+} ionic migration into the cytoplasm. In this phase, an anionic phospholipid (cardidipin) was dissociated from cytochrome C as the result of Bax protein binding. After cytochrome C is liberated, caspase-3 is activated by caspase-9, which is promoted by the cleavage of poli-ADP-ribose polymerase (PARP) enzyme [42].

Phomopsis sp. is an endophytic fungi that usually available in human being [43]. Based on living characteristics, endophytic fungi from the soursop leaf has high similarity to *Phomopsis* sp. The similarity indicates a similar evolutionary pathway. The molecular identification data based on ITS gene of soursop leaf endophytic fungi indicates the isolate is a *Phomopsis* sp. species.

We conclude that the ethyl acetate extracts of soursop-leaf-endophytic fungi are cytotoxic to breast cancer cells by inhibiting their proliferation without affecting the metabolism of normal breast cells. Cytotoxic tests showed that the extract Sir-G5 (from Garut, West Java) has the highest activity with an IC_{50} value of 19.20 μg/mL. Molecular identification based on rRNA ITS gene indicated that the soursop leaf endophytic fungi has high similarity with *Phomopsis* sp. species with homology value of 99.998%.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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