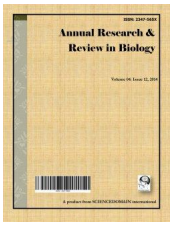

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Anticancer Activities of Endophytic Fungi Isolated from Soursop Leaves (*Annona muricata* L.) against WiDr Cancer Cells

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to investigate the *in vitro* toxicity activity of ethyl acetate extracts of endophytic fungi isolated from soursop leaves (*Annona muricata* L.) against colon cancer and analyse the bioactive compounds from the fungal extract with best anticancer activity using Gas Chromatograph-Mass Spectrometer (GC-MS).
Study Design: The experimental design used was a completely randomized factorial design with two factors, the type and concentration of endophytic fungi extract.
Place and Duration of Study: Industrial Microbiology Laboratory of Biology Research Center,

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Indonesian Institute of Sciences (LIPI) Cibinong-Bogor, Laboratory of Microbiology and Immunology of Center for Animal and Primate Studies of Bogor Agricultural University and Center of Forensic Laboratory, conducted from March 2016 to March 2017.

Methodology: Cytotoxic property was determined using 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay method. Entophytes were extracted using ethyl acetate solvent. Extract of ten isolates (at concentration of 100 µg/mL) were examined for the inhibition effect to WiDr cancer cells. Five isolates that showed significantly high inhibition effect ($P<0.05$) were further selected for IC_{50} determination against WiDr cell lines at extract concentration of 25, 50, 100, 200 and 400 µg/mL. Three isolates that significantly showed high activity ($P<0.05$) were chosen, and then measured for their toxicity (IC_{50} value) to normal cell (Chang cell lines) at concentration of 25, 50, 100, 200 and 400 µg/mL. The bioactive compound of the isolate which had the lowest toxicity to normal cell was then analysed using GC-MS.

Results: Fungal extracts of ten isolates were examined for their cytotoxicity to WiDr cancer cells with the result showed that isolate of Sir-CA1, Sir-CA2, Sir-G2, Sir-G4 and Sir-SM2 had high inhibitions. From the five fungal extracts, isolate of Sir-CA1, Sir-G2 and Sir-SM2 showed high cytotoxicity to WiDr cancer cell. Among the three isolats, fungal extract of isolate from Sukabumi (Sir-SM2) showed the lowest toxicity to normal cell was analysed on secondary metabolite compounds using GC-MS. The fungal extract contained ester group, alkaloid, saturated fatty acid, unsaturated fatty acid, terpene, terpenoid and aromatic compounds.

Conclusion: Ethyl acetate extracts of endophytic fungi Sir-SM2 isolated from soursop leaves (*Annona muricata* L.) had high cytotoxic effect on colon cancer cell (IC_{50}=20.80 µg/mL) and the lowest toxicity to normal cell compared with other fungal extracts (IC_{50}=63.69 µg/mL). The analysis of bioactive compounds with GC-MS showed that fungal extract of isolate Sir- SM2 contained compounds such as alkaloid, saturated fatty acid, unsaturated fatty acid, terpene and terpenoid that had function as anticancer.

Keywords: Annona muricata L.; endophytic fungi; metabolite; WiDr; Chang; GC-MS.

1. INTRODUCTION

The mechanism of inhibition or treatments to cancer is very complex. Lately, there have been many studies that reveal the mechanisms in inhibition of tumor or its healing. Treatments commonly used in cancer patients include surgery, chemotherapy, radiation therapy, targeted therapy and immunotherapy [1]. Side effects arising from cancer treatment are quite diverse, including hair loss, premature monopause, fatigue, infection, mouth and throat injuries, memory problems and so on [2]. Treatment using herbal and phytochemical compounds derived from plants is known as an equally effective treatment step. Several clinical studies had reported a beneficial effect of herbal treatment in survival and immune modulation of cancer patients [3].

Cancer diseases have many types and some of them are colorectal cancer and cervical cancer. Colorectal cancer is a type of cancer suffered by men and women with a high prevalence of death in Indonesia after lung cancer and liver cancer of 10.2% in men and of 8.5% in women respectively [4]. Colorectal cancer itself is divided into two, those are colon cancer and rectum cancer. According to the American Cancer Society (2017) the number of colon cancer patients is much greater than patients of rectum cancer [5].

Medicinal plants that contain secondary metabolite compounds and show significant antioxidant activity may play an important role for the treatment of cancer [6]. Secondary metabolite compounds are isolated from the plants, fungi, bacteria and many other organisms. Endophytic microbes such as fungi and bacteria have been a source of various bioactive compounds [7]. Plant endophytic fungi have been recognized as novel resources of natural bioactive products, especially in production of anticancer compounds [8]. These bioactive compounds showed cytotoxic activitiy against some types of cancer lines [9,10]. Many studies had been done to investigate the potential of endophytic fungi as cytotoxic agents against cancer cell lines.

Study of Astuti et al. [11] demonstrated the ability of three endophytic fungi (namely DP2, DP6 and E2) isolated from *Artemisia annua* L. to inhibit

WiDr cell proliferation with IC_{50} values of 332.9, 128.9, 79.8 µg/mL respectively. Another study of Astuti et al. [11] informed that the endophytic fungus namely BS1 had showed the inhibtion to WiDr cell line with IC_{50} of 120.38 µg/mL [12].

Soursop plant (*Annona muricata* L.) is widely distributed in the mainland south asia and southeast asia including Indonesia and has been used traditionally for treatment of various diseases. This is one of herb plants that contains bioactive compounds as antioxidant and its bioactive compounds play an important role in the treatment of cancer [13]. According to study of Baskar et al. [14] that soursop leaf (*Annona muricata* L.) had strong antioxidant activity of 70 µg/mL. Various studies had been conducted on the use of bioactive compounds as antioxidant and can be linked to the decrease of cancer in humans [14].

The production of bioactive compounds through microbial endophytes isolated from plant are also potential to be developed into drugs [15]. With the knowledge of the potential of the active compound from endophyt fungi, the use of this compounds as anti-cancer drugs will be widely open without having to use soursop fruit in large quantities.

Previous study of Minarni [16] showed activity from endophytic fungal extracts isolated from soursop leaves (*Annona muricata* L.) were potential as breast anticancer MCF-7 with best IC_{50} value of 19.20 ± 7.71 µg/mL, so further investigation was needed on the effect of cytotoxic extracts from endophytic fungi isolated from soursop leaf against other cancer cells [16]. This study aimed to identify the cytotoxic activity of endophytic fungal extract on colon cancer cell (WiDr) and normal cell (Chang) in vitro and to analyse the compound content from the endophytic fungal extract with the best activity.

2. MATERIALS AND METHODS

2.1 Source of Endophytic Fungi

Ten isolates of endophytic fungi were obtained from Rahman's research [17] from three districts in Java Island of Indonesia, those were from Cianjur (Sir-CA1, Sir-CA2, Sir-CA3), Garut (Sir-G2, Sir-G3, Sir-G4, Sir-G5) and Sukabumi (Sir-SM1, Sir-SM2, Sir-SM3).

2.1.1 Cultivation of endophytic fungi

The fungi culture maintained in Malt Extract Glucose Yeast Extract Peptone Agar (MGYPA) medium aseptically and then incubated at 29°C for two days. Bioactive compound and secondary metabolite were obtained from stationary phase of each isolates in liquid culture, by culturing each fungi in Malt Extract Glucose Yeast Extract Peptone Broth (MGYPB) medium and incubated at 29°C for 21 days [18,19].

2.1.2 Extraction metabolite compounds of endophytic fungi

Bioactive compounds were extracted using ethyl acetate as organic solvent according to Minarni [16]. Endophytic fungi that had been cultivated for 21 days were then extracted to obtain the main compounds. Fungi grown in 150 mL of MGYPB medium that were added 150 mL distillate of ethyl acetate solvent, then shuffled manually for 30 min. The top layer of the fraction was poured into a boiling flask and evaporated using a rotary vacuum evaporator at 36°C then the concentrated extractdried with a stream of nitrogen gas. Crude extracts obtained were stored at 3°C until analyses [20].

2.1.3 Cytotoxic activity

The cytotoxic potential of organic extracts derived from the fermented broths of individual endophytes was tested against two cell lines i.e. Colon cancer cell lines (WiDr, ATCC®-CCL ™ 218) and Normal liver cell line (Chang, ATCC®-CCL ™ 13). Both of the cell lines were cultured in cell growth medium RPMI-1640 (Roswell Park Memorial Institute) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, then maintained at 37°C in 5% of an atmospheric CO_2. About 100 µL of media containing $5x10^3$ cells was added to 96-well plate and incubated for 48 h until 70%-80% confluent. The negative control used for the initial assay was cancer cells without treatment, while the positive control was cancer cells injected with doxorubicin solution at concentration of 6 µg/mL. Fungal extracts of ten isolates were tested using concentration of 100 mg/mL to WiDr cancer cell. Both control and tested fungal extracts were inoculated in 96 well plate and incubated at 37°C for 24 hour. Cytotoxic assay was conducted using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and dissolved in 1x Phosphat Buffered Saline (PBS). The absorbance of living cell was measured

using an Elisa reader type Benchmark Bio-Rad at wavelength of 595 nm. Five isolates with highest inhibition were chosen for further cytotoxic assay to WiDr cancer cell at various concentrations of 25, 50, 100, 200 and 400 µg/mL respectively to obtain IC_{50} value. The fungal extracts of five isolates with the highest cytotoxity to cancer cell then were tested for their toxicity to normal cells (Chang cell) at various concentrations of 25, 50, 100, 200 and 400 µg/mL respectively to obtain IC_{50} value. The concentration of extracts required to kill 50% of cell population (IC_{50}) was determined from data generated by plotting a dose-response curve [12,21].

2.1.4 Gas chromatography-mass spectrometry (GC-MS) analysis

The crude of ethyl acetate extract of endophytic fungi with the high toxicity to WiDr cell and the lowest toxicity to Chang cell was was analyzed using Agilent 5973 gas chromatograph equipped with Agilent 5973 MSD detector to identify the bioactive compounds. The sample was injected via an all-glass injector working in the split mode, with Helium as the carrier gas with a flow rate of 1.0 mL/min and injection volume of 1 µL (ratio 10: 1). The Agilent 19091S-436 HP-5MS fused silica capillary column (Length – 60 m; Film thickness- 250 µm; I.D 0.25 µm) was used. Injection temperature at 290°C with split mode and pressure at 18.38 psi. The GC MS was maintained to detect compound with average size of 40-500 mass. MS conditions were with MS quad temperature of 150°C and MS source temperature of 250°C. The mass spectrum was taken at 70 eV with a scan interval of 0.5 second. The GC-MS mass spectrum interpretation was performed using WILEY and NIST (National Institute of Standards and Technology) databases [20,22].

2.2 Statistical Analysis

The experimental design used in analyzing the results of this study was a Completely Randomized Design (CRD) with two factorials the type of ethyl acetate extract of endophytic fungi and variation concentrations of extract. The data obtained were analyzed using one-way ANOVA. A significant difference between the extracts was assessed by the Tukey test with 95% confidence level. All data were expressed as mean ± standard deviation (SD)(number of replicate=3) with Pvalue<0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1 Extract of Endophytic Fungi

Identification of ten fungal isolates from previous research [17], was done based on macroscopic characteristics, and morphological identification included color of colony, texture of colony, edge of colony and diameter size of colony [16] and also using molecular identification based on ITS (Internal Transcribed Spacer) ampilfication. The extraction of endophytic fungi was expected to produce crude extracts containing anticancer compounds. Ethyl acetate was choosen as solvent for extraction of anticancer compounds, as this solvent known has low toxicity, easy to evaporate and has semi polar character. Semi polar character could bind both polar and non polar compound of fungal extract [23,24]. As reported by Putri et al. [25] that ethyl acetat solvent could extract various compounds such as alkaloid, flavonoid, saponin, tannin, polyphenol, and triterpenoid group compound from mangosteel peel extract.

Then isolates of endophytic fungi produced different amount of extract from 11 mg to 35 mg from 150 ml of broth culture medium as shown in Tabel 1. This variation due to the different bioactive compounds contained in the each endophytic fungus [26,27]. It is reported that endophytic fungi from the same host plat, could contain identical bioactive compound but shown different activity [6]. Table 1 show the production of endophytic extract from ten endophytic isolates using ethyl acetate solvent.

Table 1. The extract result obtained from endophytic fungi isolated from soursop leaves

No	Sample	Weight (mg)
1	Sir-CA1	14
2	Sir-CA2	35
3	Sir-CA3	11
4	Sir-G2	16
5	Sir-G3	13
6	Sir-G4	28
7	Sir-G5	31
8	Sir-SM1	12
9	Sir-SM2	20
10	Sir-SM3	15

3.2 Cytotoxic Activity to WiDr Cell Lines

The reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagents by

the enzyme succinic dehydrogenase in living cells is able to be relied upon to check for cell proliferation of cancer cell [28]. Ten isolates of endophytic fungi showed anticancer activity as shown in Fig. 1. The five highest anticancer activity were isolate of Sir-CA1, Sir-CA3, Sir-G2, Sir-G4 and Sir-SM2 (Fig. 1).

The WiDr cell is cancer cell with a high expression of cycolooksigenase-2 (COX-2) that promotes excessive cell proliferation [29]. COX-2 is induced by cytokines, growth factors and other agents [30]. Anticancer agents in the extracts of endophytic fungi were able to induce apoptosis of cell cancer. That early cell apoptosis undergoes a series of morphological changes in which a plasma membrane leak occurs.

Extracts of endophytic fungi had the ability to induce apoptosis of cancer cell that was caused by some metabolite compounds produced such as hexahydro- pyrrolo [1,2-a] pyrazine-1,4-dione and hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione as alkaloid compound. Hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione been reported in study of Lalitha et al. [31] that it could induce apoptotic morphological changes and DNA fragmentation in the cancer cells, which indicated that this alkaloid compound induced apoptosis in A549 and HeLa cancer cells. Hexahydro-pyrrolo[1,2-a]pyrazine-1,4- dione was also known had the strong antioxidant activities [32,33]. Antioxidant had been evaluated could induce DNA double-strand breaks and led to apoptosis [34]. The decrease in cell viability and cell proliferation in MTT testing showed induction of apoptotic activity. Morphological changes in WiDr cancer cells according to Ota et al. [30] that morphological changes were closely related to anticancer or cytotoxic activity. Morphological changes were seen in WiDr cancer cells by comparing cells with treatment by extracts and without treatment [35]. The WiDr cell in the controlled treatment was round, protected by a clear cell wall and glowed under a microscope while after being treated undergoes morphological changes to be smaller or shrunken with lower cell density. WiDr cancer cells that were given positive control treatment of doxorubicbin showed all cells became shrunken.

Then the five extracts With the highest inhibition on WiDr cancer cell were re-tested using five concentration variations of 25, 50, 100, 200 and 400 µg / mL. The result of inhibition absorbance test of cytotoxic activity extract was then calculated to find IC_{50} value (Table 1). The value of IC_{50} is the concentration of extract required to inhibit the growth of cancer cells by 50% [36].

Table 2. IC_{50} value of ethyl acetate extract of endophytic fungi on the WiDr cell

Sample	IC_{50} (µg/mL)
Sir-CA1	8.82 ± 0.23^{c}
Sir-CA2	79.98 ± 7.21^{a}
Sir-G2	8.48 ± 0.15^{c}
Sir-G4	41.02 ± 7.42^{b}
Sir-SM2	20.80 ± 4.12^{c}

Different letters show significant differences in the Tukey test (P<0.05)

Based on the the American National Cancer Institute in the study of Itharat et al. [37] that a crude extract for preliminary assay has the criteria of cytotoxicity activity (capable of inhibiting 50% of cancer cell population) if it has an IC_{50} value <30 µg / mL (at concentrations below 30 ppm).

The results showed that Sir-CA1,Sir-G2 and Sir-SM2 extracts had strong cytotoxic activity against WiDr cells with IC_{50} values of 8.82 ± 0.23 µg/mL, 8.48 ± 0.15 µg/mL and 20.80 ± 4.12µg/mL respectively. Based on study of Fitri [38] that ethanol extract of soursop leaves has anticancer activity against WiDr cells with IC_{50} value of 5.573 ± 1.60 µg/mL. Sir-G2 and Sir-CA1 extracts showed best toxicity activity against colon cancer cells as well as Fitri's study. The value of IC_{50} Sir-SM2 was also evaluated as fungal extract with high toxicity was less than 30 µg/mL.

Cytotoxic activity of ethyl acetate extract from soursop leaf against colon cancer cell lines has not been widely reported.

3.3 Cytotoxic Activity to Chang Normal Cell

One of desired criteria for new anticancer agent is low toxicity against Normal cells [39]. Therefore the chosen isolates were Sir-CA1, Sir-G2, and Sir -SM2, then tested to normal cell line (Chang cell) (Table 3). Aceptable IC_{50} value of new anticancer compound is less than 30 µg/mL for cytotoxicity screening of crude plant extracts [37]. Fungal extract of isolate Sir -SM2 gave IC_{50} value for anticancer activity of 20.80 µg/mL and compared to isolates of Sir-CA1 and Sir-G2, isolate Sir -SM2 gave the lowest toxicity for normal cell (IC_{50} = 20.80 µg/mL).

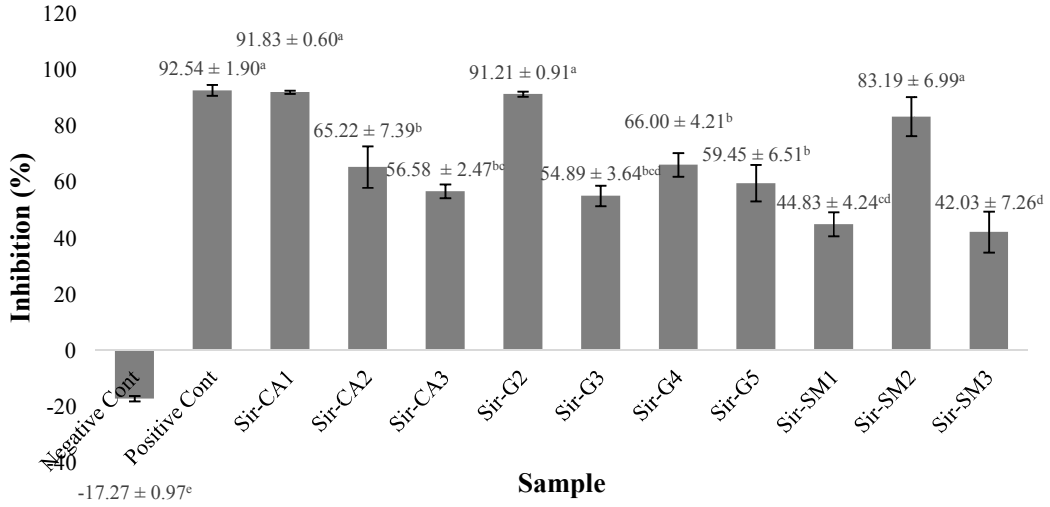

Fig. 1. Cytotoxic activity of endophytic extract to WiDr cells at concentration of 100 µg / mL

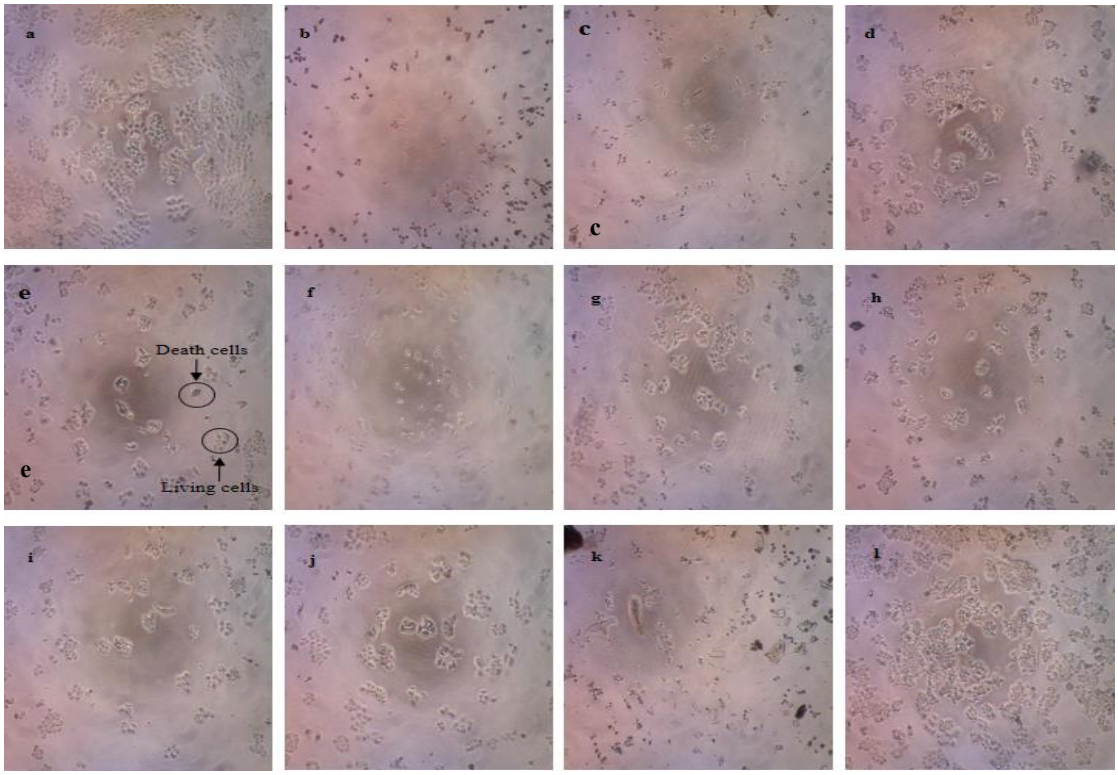

Fig. 2. Morphology of colon cancer cell lines (WiDr cell) after treatment with extract of endophytic fungi isolated from soursop (*Annona muricata* L.) leaves at concentration of 100 µg/mL

Description: a = control without treatment, b = doxorubicin as control positive, c = Sir-CA1 extract treatment, d = Sir-CA2 extract treatment, e = Sir-CA3 extract treatment, f = Sir-G2 extract treatment, g = Sir-G3 extract treatment, h = Sir-G4 extract treatment, i = Sir-G5 extract treatment, j = Sir-SM1 extract treatment, k = Sir-SM2 extract treatment, l =Sir-SM3 extract treatment (observed by canon microscope Inferted, Dyno Eye camera size 1280x1024 units: inch) at 10x magnification)

Table 3. IC_{50} value of ethyl acetate extract of endophytic fungi on the Chang cell

Sample	IC_{50} (µg/mL)
Sir-CA1	8.23 ± 0.14^{c}
Sir-G2	23.65 ± 0.65^{b}
Sir-SM2	63.69 ± 6.25^{a}

Different letters show significant differences in the Tukey test (P<0.05)

These data indicated that Sir-SM2 extract was actively inhibit/ kill colon cancer cells, but had smallest toxicity for normal cells compared with extracts from Cianjur and Garut.So Sir-SM2 was chosen as the best isolate for the further analysis of Gas Chromatography-Mass Spectrometry (GC-MS).

3.4 GC-MS Analysis

The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST and WILEY library. The compounds were tentatively identified on the basis of the NIST and WILEY data base by virtue of comparisons made of the actual mass spectral data acquired on each compound to the data base [40]. The chromatogram and the spectral analysis along with the name, molecular weight and structure of the components of the endophytic fungi were shown below in Fig. 3. Forty-six peaks were detected on the chromatogram of GC-MS analysis with retention time of 5.747 to 20.74 minutes with different quality. Eighteen high-quality chemical compounds (quality ≥ 80) are bioactive compounds contained in the extract.

Eighteen compounds can be classified into carboxylic acids (*3-methyl-2-butenoic acid and n-Hexadecanoic acid*), aromatic compounds (*5-ethyldihydro-2(3H)-Furanone and 1-amino-2-methyl-9,10-Anthracenedione*), alcohol (*D-Fenchyl alcohol*), terpenoids (*1,7,7-trimethyl-Bicyclo[2.2.1]heptan-2-one*), alkanes (*1,3-dimethyl-1-cyclohexene*), terpene (*α, α, 4-trimethyl-(S)-3-cyclohexene-1-methanol*), alkaloids *hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-Pyrrolo [1,2-a] pyrazine-1, 4-dione, 2-tert-Butylquinoline and 4-(3-Methyl-2-butenyl)-1H-indole*), saturated fatty acid (*14-methyl-Pentadecanoic acid*), unsaturated fatty acids (*9,12-Octadecadienoic acid (Z,Z)*), esters (*2-depentyl-Perhydro-htx-2-one*) and aldehydes (*Acridine-9-carbaldehyde*), ketones (*(E,E)-2-methyl-6-oxo-2,4-heptadienal and 3-Hydroxy-4-methoxyacetophenone*).

Six compounds have anticancer activity such as *hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione* (with abundance of 1.59%) [41, 42], *hexahydro-Pyrrolo [1,2-a] pyrazine-1, 4-dione* (2.56%) [31,42,43], *n-hexadecanoic acid* (3.44%) [44-46], *9,12-Octadecadienoic acid (Z,Z)*(1.51%) [47-50], *1,7,7-trimethyl-Bicyclo[2.2.1]heptan-2-one* (0.34%) [51-53] and *α, α, 4-trimethyl-(S)-3-cyclohexene-1-methanol* (0.69%) [54-56]. These bioactive compounds belong to a group of alkaloid, carboxylic acids, unsaturated fatty acids, terpenoids and terpenes compounds.

Other bioactive compounds such as *14-methyl-pentadecanoic acid* (0.41%) have antioxidant activity [57], *acridine-9-carbaldehyde* (2.62%) with its ability as an anticonvulsive drug [58], *3-hydroxy-4-methoxyacetophenone* (3.69%) had analgesic effects [59], *(E, E) -2-methyl-6-oxo-2,4-heptadienal* (29.29%), *D-Fenchyl alcohol* (0.36%) and *5-ethyldihydro- 2 (3H) -Furanone* (1.65%) used as ingredients in foodstuff [60-62]. There were several compounds that had not reported on the activity in the literature of studies such as *3-methyl-2-butenoic acid* (2.29%), *1,3-dimethyl-1-cyclohexene* (0.66%), *2-tert-Butylquinoline* (2.14%), *4- (3-Methyl -2-butenyl) -1H-indole* (1.26%), *2-depentyl-Perhydro-htx-2-one* (0.44%) and *1-amino-2-methyl-9,10-Anthracenedione* (2.78%).

This study provided evidence for cytotoxicity in WiDr and Chang cell lines which may be due to existing phytochemicals in the extract as mentioned previously. Alkaloid compounds (*hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione* and *hexahydro-Pyrrolo [1,2-a] pyrazine-1, 4-dione*) in endophytic fungal extract of isolate Sir-SM2 have alkylating activity that can cause DNAstrand breakage and damage, leading to the cells death not only to cancer cell but also normal cell. These IC_{50} data are interesting as it suggests that the fungal extract is more toxic for cancer cells than on normal cells. An crude extract is considered safe when having IC_{50} is more than the provision limit set by American National Cancer Institute (30 µg/ml) [63]. For the best result of fungal extract as anticancer agent against cancer cell lines without damaging normal cells, the dosages, the routes of administration and the treatment procedures are very important for the further investigation. The transformation of chemical structures and the application of new drug delivery systems may reduce the toxicities of these extract compounds particularly for

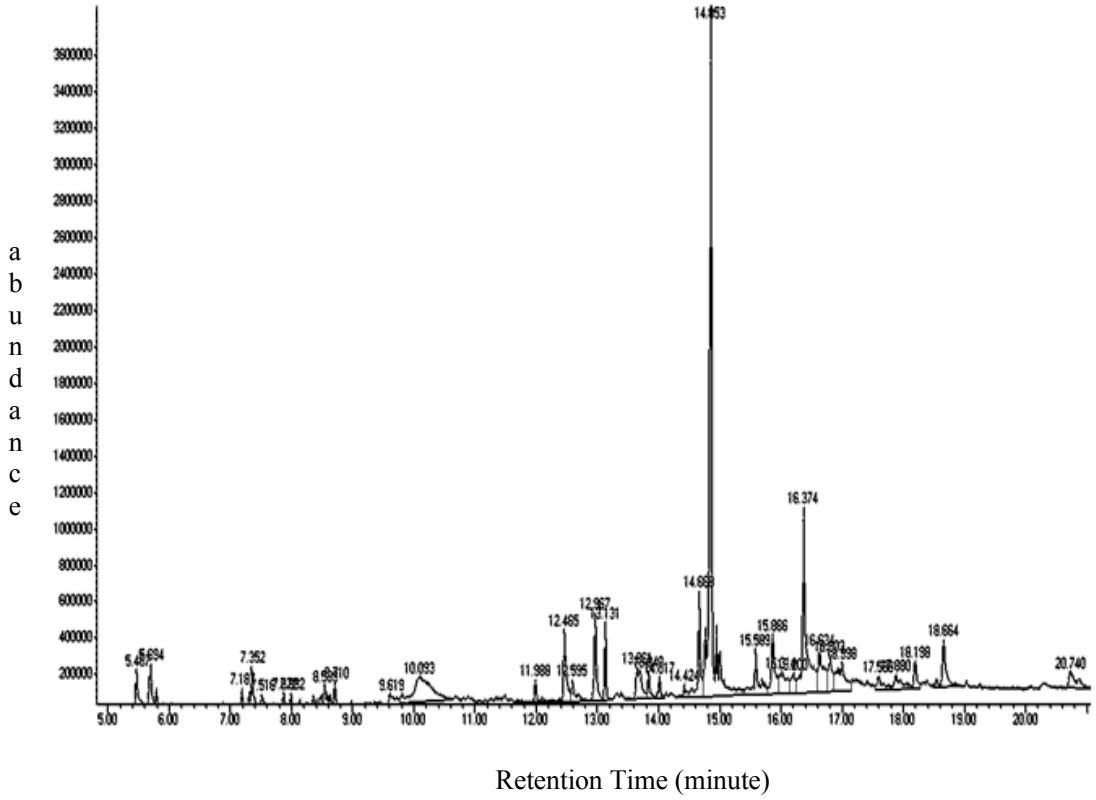

Fig. 3. GC/MS Chromatogram of ethyl acetate extract of endophytic fungi isolated from ursop (*Annona muricata* L.)

normal cell line [64]. The isolation and use of selective cytotoxic compounds to cancer cells can be of pharmacological importance for the further investigation of this study.

4. CONCLUSION

This study proved that crude ethyl acetate extract of endophytic fungi Sir-SM2 isolated from soursop (*Annona muricata* L.) leaf obtained from Sukabumi was potential as natural anticancer which had high cytotoxic effect on colon cancer cell and low toxicity effect on normal cell. The compounds of fungal extract of isolate Sir-SM2 identified by GC-MS analysis contained some compounds which had antioxidant activity from *14-methyl-pentadecanoic acid* compound and *hexahydro-pyrrolo[1,2-a]pyrazine-1,4- dione* and also anticancer activity from *hexahydro-3-(2-methylpropyl)-Pyrrolo[1,2-a]pyrazine-1,4-dione*, *hexahydro-Pyrrolo [1,2-a] pyrazine-1, 4-dione*, *n-Hexadecanoic acid*, *9,12-Octadecadienoic acid (Z,Z)*, *1,7,7-trimethyl-Bicyclo[2.2.1]heptan-2-one* and *α, α, 4-trimethyl-(S)-3-cyclohexene-1-methanol*. The results showed that endophytic fungi isolated from soursop (*Annona muricata* L.) leaves could be used as a potential candidate for drug development for anticancer.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. [ACS] American Cancer Society. Treatments and side effects; 2017. Available:https://www.cancer.org/treatment/treatments-and-side-effects.html (Accessed July 2017)
2. Komen, Susan G. Komen for the cures: Chemotherapy and side effects. United

States: Health Communication Research Laboratory Saint Louis University; 2009.
3. Yin SY, Wei WC, Jian FY, Yang NS. Therapeutic applications of herbal medicines for cancer patients. Evid Based Complement Alternat Med. 2013;1-15.
4. [WHO] World Health Organization. Cancer country profiles; 2014. Available:http://www.who.int/cancer/country-profiles/en/ (Accessed 8 May 2017)
5. [ACS] American Cancer Society. Key statistics for colorectal cancer; 2017. Available:https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics/ (Accessed 11 May 2017)
6. Kaur R, Kapoor K, Kaur H. Plants as a source of anticancer agents. J Nat Prod. Plant. Resour. 2011;1(1):119-124.
7. Kumar S, Aharwal RP, Shukla H, Rajak RC, Sandhu SS. Endophytic fungi: As a source of antimicrobials bioactive compounds. WJJPS. 2014;3(2):1179-1197.
8. Chen L, Zhang QY, Jia M, Ming QL, Yue W, Rahman K, Qin LP, Han T. Endophytic fungi with antitumor activities: Their occurrence and anticancer compounds. Crit Rev Microbiol. 2016;42(3):454-73.
9. Zheng CJ, Xu LL, Li YY, Han T, Zhang QY, Ming QL et al. Cytotoxic metabolites from the cultures of endophytic fungi from Panax ginseng. Appl Microbiol Biotechnol. 2013;97(17):7617-7625.
10. Cui J, Guo S, Xiao P. Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. J Zhejiang Univ-Sci B (Biomed & Biotechnol). 2011;12(5):385-392.
11. Astuti P, Wahyono, Nuryastuti T, Purwantini I, Purwanto. Antimicrobial and cytotoxic activities of endophytic fungi isolated from *Artemisia annua* L. JAPS. 2014;4(10):047-050.
12. Astuti P, Wahyono, Nababan OA. Antimicrobial and cytotoxic activities of endophytic fungi isolated from *Piper crocatum* Ruiz and Pav. Asian Pac J Trop Biomed. 2014;4(2):S592-S596.
13. Tan RX, Zou WX. Endophytes: A rich source of functional metabolites. Nat Prod Rep. 2001;18:448-459.
14. Baskar R, Rajeswari V, Kemar TS. *In-vitro* antioxidants studies in leaves of annona species. Indian J Exp Biol. 2007;45(5):480-485.
15. Strobel G. Endophytes as sources of bioactive products. Microb Infect. 2003;5:535–544.
16. Minarni. Aktivitas antikanker ekstrak etil asetat kapang endofit daun sirsak *(Annona muricata L.*.Bogor (ID): Institut Pertanian Bogor; 2015. Indonesian
17. Rahman F. Aktivitas ekstrak jamur endofit dari daun sirsak *(Annona muricata L.)* terhadap viabilitas khamir Saccharomyces cerevisiae dan Candida tropicalis. Bogor (ID): Institut Pertanian Bogor; 2015. Indonesian
18. Waluyo L. Teknik dan metode dasar dalam mikrobiologi. Malang (ID): UMM Press; 2008. Indonesian
19. Agusta A. Nerolidol, komponen kimia aromatic tanaman teh yang juga diproduksi oleh jamur endofit *Schizophyllum* sp. D. Berita Biologi. 2013;12(2):77-181. Indonesian
20. Devi NN, Prabakaran JJ. Bioactive metabolites from an endophytic fungus *Penicillium* sp. Isolated from *Centella asiatica*. J Cream. 2014;4(1):34-43.
21. Hasan AEZ, Mangunwidjaja D, Sunarti TC, Suparno O, Setiyono A. Investigating the antioxidant and anticytotoxic activities of propolis collected from five regions of Indonesia and their abilities to induce apoptosis. Emir. J. Food Agric. 2014;26(5): 390-398.
22. Mtunzi F, Dikio D, Makhwaje A, SipamLa, Modise SJ. GC-MS analysis of hexane extract of *Bolusanthus speciosus* stem bark. Asian Plant. 2013;3(2):27-30.
23. [GPS] Global Product Strategy. Ethyl acetate. Belgia (BE): Solvay Company; 2012.
24. Firdiyani F, Agustini TW, Ma'ruf WF. Ekstraksi senyawa bioaktif sebagai antioksidan alami S*pirulina platensis* segar dengan pelarut yang berbeda. JPHPI. 2015;18(1):28-37. Indonesian
25. Putri WS, Warditiani NK, Larasanty LPF. Skrining fitokimia ekstrak etil asetat kulit buah manggis (*Garcinia mangostana* L.). J Farmasi Udayana. 2013;2(4):56-60. Indonesian
26. Senthilkumar N, Murugesan S, Babu DS. Metabolite profiling of the extracts of endophytic fungi of entomopathogenic significance, *Aspergillus flavus* and *Nigrospora sphaerica* isolated from tropical tree species of India, *Tectona grandis* L. J Agric Life Sci. 2014;1(1):108-114.

27. Nicoletti R, Fiorentino A. Plant bioactive metabolites and drugs produced by endophytic fungi of Spermatophyta. Agriculture. 2015;5:918-970.
28. [ATCC] American Type Culture Collection. MTT cell proliferation assay; 2017. Available:https://www.atcc.org/~/media/DA5285A1F52C414E864C966FD78C9A79.ashx/ (Accessed 17 March 2017)
29. Palozza P, Serini S, Maggiano N, Giuseppe T, Navarra P, Ranelletti FO. β-Carotene downregulates the steady-state and heregulin-a-induced cox-2 pathways in colon cancer cells. J. Nutr. 2005;135:129-136.
30. Ota S, Bamba H, Kato A, Kawamoto C, Yoshida Y, Fujiwara K. Review article: COX-2, prostanoids and colon cancer. Aliment Pharmacol Ther. 2002;16(2):102-106.
31. Lalitha P, Veena V, Vidhyapriya P, Lakshmi P, Krishna R, Sakthivel N. Anticancer potential of pyrrole (1, 2, a) pyrazine 1, 4, dione, hexahydro 3-(2-methyl propyl) (PPDHMP) extracted from a new marine bacterium, *Staphylococcus* sp. strain MB30. Apoptosis. 2016;21(5):566-77.
32. Gopi M, Dhayanithi NB, Devi KN, Kumar TTA. Marine natural product, Pyrrolo [-a] pyrazine–dione, hexahydro-(C7H10N2O2) of antioxidant properties from Bacillus species at Lakshadweep archipelago. J. Coastal Life Med. 2014;2:632–637.
33. Balakrishnan D, Bibiana A, Vijayakumar A, Santhosh R, Dhevendaran K, Nithyanand P. Antioxidant activity of bacteria associated with the Marine Sponge *Tedania anhelans*. Ind. J. Microbiol. 2015;5:13–18.
34. Lu LY, Ou N, Lu Q. Antioxidant induces dna damage, cell death and mutagenicity in human lung and skin normal cells. Sci. Rep. 2013;3(3169):1-11.
35. Kusuma AW, Nurulita NA, Hartanti D. Efek sitotoksik dan anti proliferative kuersetin pada sel kanker kolon WiDr. Pharmacy. 2010;7(3):107-122. Indonesian
36. Boyd MR, Paull KD, Rubinstein LR. Data display and analysis strategies for the NCI disease-oriented *in vitro* antitumor drug screen. In Valeriote FA, Corbett T, Baker L (eds): Cytotoxic anticancer drugs: Models and concepts for drug discovery and development. Amsterdam (NL): Klower Academic Publishers; 1992.
37. Itharat A, Houghton PJ, Eno AE, Burke PJ, Sampson JH, Raman A. *In vitro* cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. J Ethnopharmacol. 2004;90:33-38.
38. Fitri RK. Uji aktivitas antikanker ekstrak daun sirsak (Annona muricata Linn.) terhadap beberapa sel kanker manusia secara in vitro. Surabaya (ID): Universitas Airlangga; 2014. Indonesian
39. Ganiswara, Nafrialdi. Farmakologi dan terapi. Edisi IV. Jakarta (ID): Fakultas Kedokteran UI; 1995. Indonesian
40. Ahmadi NR, Mangunwidjaja D, Suparno O, Pradono DI. Extraction process optimization of kamandrah (*Croton tiglium* L.) seed with expression and identification of active ingredient as botanical larvacide of dengue fever preventive. J Tek Ind Pert. 2012;21(3):154-162.
41. Melo IS, Santos SN, Rosa LH, Parma MM, Silva LJ, Queiroz SC, Pellizari VH. Isolation and biological activities of an endophytic *Mortierella alpina* strain from the Antarctic moss *Schistidium antarctici*. Extremophiles. 2014;18(1):15-23.
42. Ser HL, Palanisamy UD, Yin WF, Chan KG, Goh BH, Lee LH. *Streptomyces malaysiense* sp. nov.: A novel Malaysian mangrove soil actinobacterium with antioxidative activity and cytotoxic potential against human cancer cell lines. Sci Rep. 2016;6(24247):1-12.
43. Malash MA, El-Naggar MM, El-Hassayeb HEA, Ibrahim MS. Production of antimicrobial Pyrrol-derivatives acting against some fish pathogens from marine MMM. Glob. Veterinaria. 2016;17(6):495-504.
44. Arunkumark V, Paridhavi M. Evaluation of the components and antimicrobial activity of volatile oil from *Zanthoxylum limonella* fruit. Int J Pharm Bio Sci. 2013;4(2):777-787.
45. Parthipan B, Suky MGT, Mohan VR. GC-MS analysis of phytocomponents in *Pleiospermium alatum* (Wall. ex Wight & Arn.) Swingle, (Rutaceae). J Pharmacog Phytochem. 2015;4(1):216-222.
46. Aparna V, Dileep KV, Mandal PK, Karthe P, Sadasivan C, Haridas M. Anti-inflammatory property of n-Hexadecanoic acid: Structural evidence and kinetic

assessment. Chem Biol Drug Des. 2012;80:434–439.
47. Arab A, Akbarian SA, Ghiyasvand R, Miraghajani M. The effects of conjugated linoleic acids on breast cancer: A systematic review. Adv Biomed Res. 2016;5:115.
48. Rajeswari G, Murugan M, Mohan VR. GC-MS analysis of bioactive components of *Hugonia mystax* L. (Linaceae). J Pharm Biomed Sci. 2013;29(29):818–824.
49. Pariza MW, Park Y, Cook ME. The biologically active isomers of conjugated linoleic acid. Prog Lipid Res. 2001;40:283-298.
50. Ryder JW, Portocarrero CP, Song XM, Cui L, Yu M, et al. Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. Diabetes. 2001;50:1149-1157.
51. Salman AS, Farghaly AA, Donya SM, Shata F. Protective effect of *Cinnamomum camphora* leaves extract against atrazine induced genotoxicity and biochemical effect on mice. J American Sci. 2012;8(1):190-196.
52. Edris AE. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: A review. Phytother Res. 2007;21(4):308–23.
53. Zainudin Z, Azim MKM. Study on the antibacterial and antifungal effects of camphor oil; *Cinnamomum camphora* with emphasis on the control of overpopulation of tilapia. IJFAS. 2015;2(5):337-340.
54. Budiman A, Arifta TI, Diana, Sutijan. Continuous production of α-Terpineol from α-Pinene isolated from Indonesia crude turpentine. Mod App Sci. 2015;9(4):225-232.
55. Gohar YM, El-Naggar MMA, Soliman MK, Barakat KM. Characterization of marine *Burkholderia cepacia* antibacterial agents. J Nat Prod. 2010;3:86-94.
56. Bintang M, Purwanto UMS, Kusumawati DE, Yang JJ. Study of endophytic bacteria as novel source of antioxidant agent based on GC-MS analysis. Int Journal Chem Envi Biol Sci. 2015;3(5):368-369.
57. Henry GE, Momin RA, Nair MG, Dewitt DL. Antioxidant and cyclooxygenase activities of fatty acids found in food. J. Agric. Food Chem. 2002;50:2231−2234.
58. Mathieu M, Dereure O, Hillaire-Buys D. Presence and *ex vivo* formation of acridone in blood of patients routinely treated with carbamazepine: Exploration of the 9-acridinecarboxaldehyde pathway. Xenobiotica. 2011;41(2):91-100.
59. Sun FZ, Cai M, Lou FC. Analgesic effect and gastro-intestinal motility inhibitory action of 3-hydroxy-4-methoxy-acetophenone from *Cynanchum paniculatum* (Bunge) Kitagawa. China J Chinese Materia Med. 1993;18:362-363.
60. Burton G, Daroszewski J. Food supplements and uses thereof. US Patents Application Publication. 2009;20090306222A1: 1-12.
61. Api AM, Belsito D, Bhatia S, Bruze M, Calow P, Dagli ML, et al. RIFM fragrance ingredient safety assessment, Fenchyl alcohol, CASregistry number 1632-73-1. Food Chem Toxicol. 2015;84:S25-S32.
62. Murat C, Bard MH, Dhalleine C, Cayot N. Characterisation of odour active compounds along extraction process from pea flour to pea protein extract. Food Res Int. 2013;53:31–41.
63. Zirihi GN, Mambu L, Guede-Guina F, Bodo B, Grellier P. *In vitro* antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria. J Ethnopharmacol. 2005;98:281-85.
64. Lu JJ, Bao JL, Chen XP, Huang M, Wang YT. Alkaloids isolated from natural herbs as the anticancer agents. J Evid Based Complementary Altern Med. 2012;485042: 1-12.

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