BIODIVERSITAS
Volume 24, Number 5, May 2023
Pages: 2716-2722

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240525

Cytotoxic screening of endophytic fungi associated with *Catharanthus roseus*

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Manuscript received: 20 March 2023. Revision accepted: 18 May 2023.

Abstract. *Lay CS, Agustina I, Astuti P, Hertiani T. 2023. Cytotoxic screening of endophytic fungi associated with* Catharanthus roseus. *Biodiversitas 24: 2716-2722.* Despite the advancements in cancer therapy, there is still a need for new anticancer agents. Due to the tremendous range of chemical compounds that endophytic fungi create, they have become a crucial component of the current drug development process. The aim of this study was to evaluate the cytotoxic potential of endophytic fungi associated with *Catharanthus roseus* (L.) G.Don collected from Yogyakarta, Indonesia. All fungal EtOAc extracts were tested against three human cancer cell lines, HeLa, T47D, MCF-7, and Vero cell lines. The potential fungal isolate was identified using molecular techniques. The results showed that a total of seven endophytic fungi were obtained from the different parts of *C. roseus*. Only one fungus (SCR 3) showed the best cytotoxic activitiy towards all three cell lines used, especially the MCF-7 cell line (IC_{50} 130.90 µg/mL, Selectivity Index=11.42). The findings of using LC-HRMS also revealed several exciting compounds such as genistein and D-(-)-Mannitol. The results showed that EtOAc extract of SCR 3 (*Aspergillus* sp.) induced apoptosis but did not affect the cell cycle. This study suggests that EtOAc extract of SCR 3 may be a potential source of secondary metabolites that can function as a new source of anticancer agents.

Keywords: *Aspergillus*, *Catharanthus roseus*, cytotoxic, endophytic fungi

Abbreviations: PDA: Potato Dextrose Agar, PDB: Potato Dextrose Broth, EtOAc: Ethyl Acetate, PCR: Polymerase Chain Reaction, DNA: Deoxyribonucleic Acid

INTRODUCTION

Catharanthus roseus (L.) G.Don (*C. roseus*), a member of the Apocynaceae family, is native to the subtropical region. This plant is endemic to Madagascar, although it is also grown in Indonesia under *Tapak Dara*. The plant produces numerous secondary metabolites, and 130 alkaloids have been discovered so far (Das and Sharangi 2017). Furthermore, this plant produces a diverse of secondary metabolites, such as chlorogenic acid, oleanolic acid, loganic acid, vindolicine, vindoline, vinblastine, vincristine, ajmalicine, and catharanthine which have pharmacologically significant effects (Barrales et al. 2019). This herb has historically used in the treatment of fever, rheumatism and fatigueness. *Catharanthus roseus* possess flavonoids, saponins, tannins, and several anticancer alkaloids (Nejat et al. 2015). Additionally, it has blood coagulation properties (Das and Sharangi 2017). The leaves of the plant are rich in the alkaloids, vincristine and vinblastine, which inhibit tumor formation (Das and Sharangi 2017). These anticancer alkaloids prevent the development of cells by adhering to microtubules, which inhibits mitosis and causes cell death (Palem et al. 2016). Significantly, *C. roseus* vinblastine and vincristine, as well as synthetic analogs, have been utilized to treat advanced testicular cancer, breast cancer, and lung cancer in combination with other cancer chemotherapeutic drugs.

The leaves of *C. roseus* are the best known source of vinblastine and vincristine; however, their yields are low. This plant is difficult and expensive to process, and takes a long time to grow (Chandra 2012), which have led to a quest for more reliable, affordable, and alternative sources of their production. Endophytic fungi are mutually connected to plants and can produce the same bioactive substances and organic molecules as their host plant. This may raise the prospect of an intergeneric genetic exchange between the two, while posing no threat to the host (Kumar et al. 2013). The host plant interacts with these microbes, and the host plant, in turn, modifies the metabolism of these endophytes to create chemicals that may have protective effects on both the microbe and the host (Kusari et al. 2012). Endophytes help sustain plant resilience to abiotic challenges, such as enhancing drought tolerance, plant disruptions during high and low temperatures, low pH environmental conditions, excessive saline, and pressure from heavy metals in the soil (Jalgaonwala et al. 2011; Sushma et al. 2021). Therefore, it is easier to determine novel compounds with promising bioactivity by screening endophytic fungi living in plant species with therapeutic characteristics (Zheng et al. 2021; Deshmukh et al. 2022; Wen et al. 2022). In addition, endophytic fungi can provide endless and practical supplies of the metabolites they create (Aly et al. 2010). Finally, due to their chemical variety, endophytic fungi are a desirable source of bioactive metabolites (Jinu and Jayabaskaran 2015).

The endophytes living inside the plant have been shown in previous investigations to generate the host-specific chemicals vincristine and vinblastine (Kumar et al. 2013; Palem et al. 2016). Additionally, it has been shown that a few new compounds with putative cytotoxic effects are produced by *C. roseus* endophytic fungus (Asai et al. 2013). So far, the endophytic colony of *C. roseus* biodiversity has been studied by many researchers. Several authors investigate endophytic colony of *C. roseus* that inhabits the northern areas of India (Kharwar et al. 2008) and the coastal regions of India (Dhayanithy et al. 2019). Hence, it is presumed that endophytic fungi living inside this plant, which are obtained from a different region, have the capacity to produce a range of pharmacologically active compounds to shield the host-plant from stress; these compounds have therapeutic properties (Rai et al. 2021). Therefore, the aim of this study was to evaluate the cytotoxic potential of endophytic fungi associated with *C. roseus* collected from Yogyakarta, Indonesia.

MATERIALS AND METHODS

Collection of plant materials and isolation of endophytic fungi

Catharanthus roseus was collected from several locations within the boundaries of Yogyakarta and identified by the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. The samples (leaves, stem, and flower) were air-dried in a laminar airflow after cleaning in running tap water, followed by surface sterilization with 70% EtOH for 2 min and NaOCl 1% for 5 min. The sterile *C. roseus* samples were cut into small pieces (about 1 cm × 1 cm for leaves and flower, 1 cm long for stem). These pieces were inoculated on PDA media (PDA + Chloramphenicol), and incubated at 25°C in dark condition. The hypha that developed on *C. roseus* leaves were moved to a fresh plate with the same PDA media and sub-cultured again to obtain a pure colony. The purity of pure culture was frequently scrutinized (Ola 2020). The grown hyphae were transferred to new petri dishes containing PDA without antibiotics until sufficient culture was obtained for fermentation.

Cultivation and extraction

Fungal isolates were cultivated in a PDB medium at 25°C for seven days after isolated from *C. roseus* plant parts. A 500 mL Erlenmeyer flask containing 200 mL of PDB medium sterilized by autoclaving at 121°C for 20 minutes and cooled down at room temperature was used for the fermentation process. Five agar plugs (6 mm diameter) of fungal colonies were inoculated in PDB medium, and the cultures were then incubated for 14 days at room temperature with 120 rpm agitation (Astuti et al. 2014). After incubation, mycelium, and broth culture were separated using filter paper. The culture medium was evaporated to reduce the volume and extracted using ethyl acetate (1:1) three times. The solvent was further evaporated and dried.

Cell proliferation assay

In this study, a microplate 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) test was used. Cytotoxicity was evaluated against T47D, MCF-7, Hela, and Vero cell lines. The DMEM medium was used to sustain cancer cells. Each well of a 96-well plate was seeded with around 4000 cells, and the cells were incubated for 24 hours. Then, for 24 hours, cancerous cells were exposed to various concentrations of fungal crude extracts (200, 100, 50, 25, 12,5 µg/mL). After treatment, 100 µL of a 5mg/mL MTT solution (made in PBS) was added to each well plate, and then incubated for 2 hours. The purple formazan crystals were dissolved in 100 µL of DMSO in each well, and absorption was calculated at 595 nm using a microplate reader.

Characterization of endophytic fungi

The potential endophytic fungus was identified to determine its species' name. The fungal isolates were identified molecularly, including DNA isolation, PCR, sequencing, Top 10 BLAST NCBI, and phylogenetic tree determined by NCBI. The sequence data were aligned using GenBank BLASTN and evaluated against sequences stored in the GenBank database. Using MEGA 6.0 software, fungal isolates phylogenetic and molecular evolutionary analysis was carried out based on their ITS sequences (Tamura et al. 2013).

Identification of bioactive compounds

The EtOAc extract of the potential endophytic fungus that showed the best cytotoxic result was analyzed for their bioactive compounds. The bioactive compounds were identified with LC-HRMS Instrument (Thermo Scientific™ Dionex™ Ultimate 3000 RSLCnano UHPLC coupled with Thermo Scientific™ Q Exactive™ High-Resolution Mass Spectrometer. Mobile phase: A=Water+0.1% Formic Acid, B=Acetonitrile+0.1% Formic acid. Analytical column: Phenyl Hexyl 100 mm x 2.1 with a flow of 0.20 mL/min, injection volume of 5 µL, and run time of 25 min. Full MS at 70,000 FWHM Resolution. Data Dependent MS2 at 17,500 FWHM. HEtOActed Electrospray Ionization (H-ESI). Compounds were identification by Thermo Scientific™ Compound Discoverer Software.

Cell cycle analysis with PI-staining flow cytometry

In this study, a total of 10^6 cancer cell cultures/wells were distributed into 6-well plates. After 24 hours of incubation, cells were treated with extract and observed the best cytotoxic activity at concentration series. After 24 hours of treatment, cells were harvested then washed with a buffer solution and incubated with trypsin solution for 2 minutes. After that cells were treated with Pl (Propidium Iodide) reagent and incubated for 10 minutes, and cell distribution was analyzed with a flow cytometer. From the flow cytometry data, the percentage of the number of cells in each cycle was obtained. The percentage of cells in each treatment was compared with the control.

Apoptosis analysis with annexin V-flow cytometry

A total of $5x10^3$ well cells were distributed to 6-well plates, then treated with the extract and incubated for 24 hours. After 24 hours, cells were harvested and washed with a buffer solution. Then, cells were re-suspended with binding buffer and added Annexin 1 FITC reagent. Flow cytometry results obtained a histogram of the distribution of viable cells, early apoptosis, late apoptosis, and necrosis.

Data analysis

The data were expressed as mean ± SD. Depending on the value of normality, the One-Way ANOVA test or the Kruskal Wallis test (SPSS software version 16) were used to assess statistical significance. P<0.05 were considered significant with a 95% confidence interval.

RESULTS AND DISCUSSION

Isolation of endophytic fungi

Results of isolation showed that a total of seven endophytic fungi were isolated from stem and leaf (3 each), and one from flower of *C. roseus*. It was found that the diversity of endophytic fungal was higher in the plant stem and leaves than in flower. Isolated endophytic fungi showed variation in colony diameter, color (white, black, and grey) and texture (velvet and cottony). Stem, flower and leaf samples showed predominantly white colonies and massive hyphae growth with yellow color (Figure 1).

Cytotoxicity screening

The EtOAc extracts of all seven fungi were tested for cytotoxic screening against T47D, MCF-7, HeLa, and Vero cell lines. The percentage of viability exhibited by EtOAc extracts of all the fungi is given in Table 1. Extract of SCR 3 showed the best cytotoxicity to three human cancer cell lines, i.e. HeLa (44.56% viability at the concentration of 200 µg/mL with selectivity index>10), T47D (63.69% viability at the concentration of 200 µg/mL) and MCF-7 (40.96% viability at the concentration of 200 µg/mL with selectivity index=11.42).

Characterization of endophytic fungi

In this study, of the seven endophytic fungal isolates, SCR 3 showed the best score for cytotoxic potential and was identified at the molecular stage. Results of molecular identification revealed that SCR 3 showed 100% percentage identity with *Aspergillus* sp. Based on the sequencing results, the top 10 BLAST NCBI and phylogenetic tree by NCBI, SCR 3 isolate was found to be similar to various species of *Aspergillus* (Figure 2).

Identification of bioactive compounds

The identified compounds of SCR3 along with their molecular weight, molecular formula, group area, and retention time in positive ion mode in ESI are presented in Table 2.

Apoptosis and cell cycle profiles of SCR 3

The EtOAc extract of SCR 3 was then assessed for apoptosis and cell cycle profiles against MCF-7 cell lines. The results exhibited that EtOAc extract induced apoptosis (up to 6.8% at IC_{50} concentration and 9.7% at $2\times IC_{50}$ concentration compared to the control) but did not modulate the cell cycle (Figures 3 and 4).

Table 1. Cytotoxicity of ethyl acetate extract against MCF-7, T47D, HeLa, and Vero cells

Cell lines	Endophytic fungal cultures extracts (% Viability)						
	LCR 1 (200 µg/mL)	SCR 2 (200 µg/mL)	SCR 1 (200 µg/mL)	LCR 2 (200 µg/mL)	SCR 3 (200 µg/mL)	FCR 1 (200 µg/mL)	LCR 3 (200 µg/mL)
MCF-7	110.3670 ± 9.09	77.0539 ± 1.63	71.4107 ± 4.6	79.3775 ± 8.08	40.9681 ± 1.66	83.0357 ± 21.46	99.7519 ± 4.89
T47D	83.1635 ± 1.11	87.4967 ± 5.35	94.44308 ± 7.14	84.5124 ± 6.54	63.3907 ± 4.42	85.7350 ± 5.75	80.7375 ± 15.33
HeLa	96.9696 ± 11.08	103.8737± 5.16	97.4175 ± 7.06	105.9301± 17.43	44.5616 ± 3.90	166.5006 ± 9.14	70.4856± 8.9
Vero	78.7511± 6.53	101.9655 ± 6.29	88.1765 ± 3.67	80.0423 ± 0.76	80.0941 ± 1.81	150.4043 ± 9.05	87.7358 ± 2.09

Note: mean ± SD of three replicates (n=3)

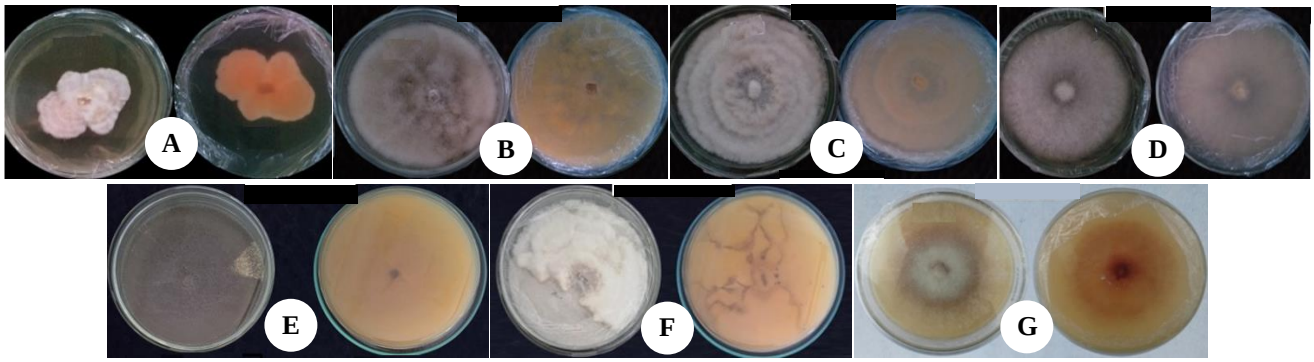


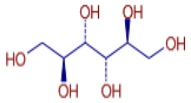
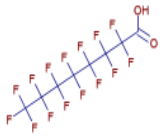
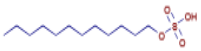
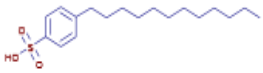
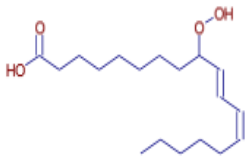
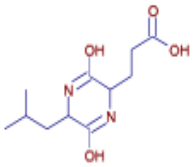
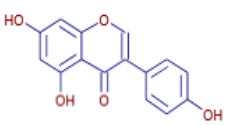
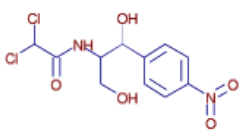
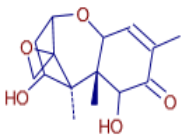
Figure 1. Endophytic fungi isolated from stem, leaves and flower of *C. roseus* on PDA. LCR (leaves isolates), SCR (stem isolates), and FCR (flower isolates). A. LCR 1, B. SCR 2, C. SCR 1, D. LCR 2, E. SCR3, F. FCR 1, G. LCR 3

Discussion

The microbiological diversity of plants, combined with the chemo diversity of the endophytic fungi, gives sufficient opportunities to discover novel lead compounds (Dhayanithy et al. 2019). The microbial-host plant connection confers the possibility for the microorganisms to develop various unique medicinally valuable products, adding to their microbial and chemical diversity. Endophytes can establish themselves in plants, and microbial-host connection often stimulates the synthesis of various metabolites by microbes in addition to compounds unique to plants (the production of which is facilitated via horizontal gene transfer) (Kusari et al. 2012).

In the present study endophytic fungi were isolated on PDA medium. Hyphae surrounding surface-sterilized plant tissue was a sign of the formation of endophytic fungal isolates. A total of seven endophytic fungal isolates were obtained from different plant parts of *C. roseus*. All fungal isolates colonies exhibit a variety of morphological traits, such as colony variation in size, color (white, black, and grey), and texture (velvet and cottony). Stem, flower and leaf samples showed predominantly white colonies and massive hyphae growth with yellow color.

Table 2. The identified compounds (Top 10) from SCR 3 extract with names, formula, calculated mass, group area, retention time (Rt), and structures

Name	Formula	Calculated MW	RT [min]	Group area: Sample	Structure
D-(-)-Mannitol	$C_6H_{14}O_6$	182.0782	0.999	5.38E+08	
Perfluorooctanoic acid (PFOA)	$C_8HF_{15}O_2$	413.9729	14.108	2.97E+08	
Dodecyl sulfate	$C_{12}H_{26}O_4S$	266.1546	15.462	2.13E+08	
4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	326.1909	17.328	1.07E+08	
Myristyl sulfate	$C_{14}H_{30}O_4S$	294.186	17.114	1.04E+08	
(+/-)9-HpODE	$C_{18}H_{32}O_4$	312.2296	14.094	10645631	
NP-016455	$C_{11}H_{18}N_2O_4$	242.126	7.291	10219697	
Genistein	$C_{15}H_{10}O_5$	270.0524	15.026	9717373	
N1-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-2,2-dichloroacetamide	$C_{11}H_{12}Cl_2N_2O_5$	322.0118	10.388	9149113	
(1'S,2'S)-3',11'-dihydroxy-1',2',5'-trimethyl-8'-oxaspiro[oxirane-2,12'-tricyclo[7.2.1.0Â?,â • ·]dodecan]-5'-en-4'-one	$C_{15}H_{20}O_5$	280.1305	11.343	4294712	

The EtOAc extracts of endophytic fungi were used to test aganist T47D, MCF-7, Hela, and Vero cell lines. With an IC_{50} of 130.90 µg/mL, it was found that EtOAc extract of SCR 3 was cytotoxic to MCF-7 cell line (Selectivity Index=11.42). EtOAc extract of SCR 3 also showed cytotoxic activity to HeLa cell line with IC_{50} 220.94 µg/mL (44.56% viability at the concentration of 200 µg/mL, SI>10) and to the T47D cell line (63.69% viability at the concentration of 200 µg/mL). Additionally, it adversely influenced Vero cells, with an IC_{50}>500 µg/mL. Furthermore, EtOAc extract of endophytic fungi (SCR 3) showed the most remarkable cytotoxicity against MCF-7. Therefore, it is hypothesized that EtOAc extract of SCR 3 contains exo-metabolites, that best exhibited cytotoxicity against MCF-7. Molecular results revealed that SCR 3 isolate was identified as *Aspergillus* sp. Based on the sequencing results, the Top 10 BLAST NCBI and Phylogenetic Tree by NCBI, SCR 3 isolate was found to be similar to various species of *Aspergillus*.

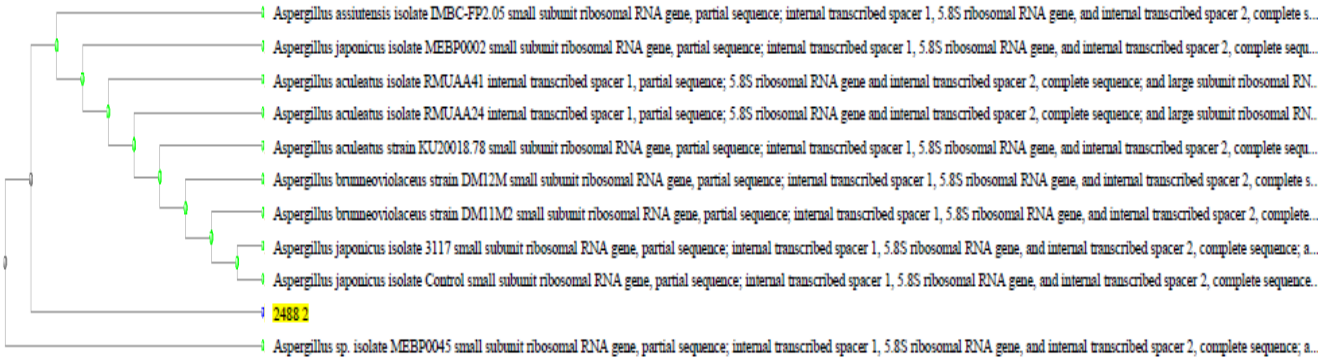


Figure 2. Phylogenetics tree of SCR 3 isolate

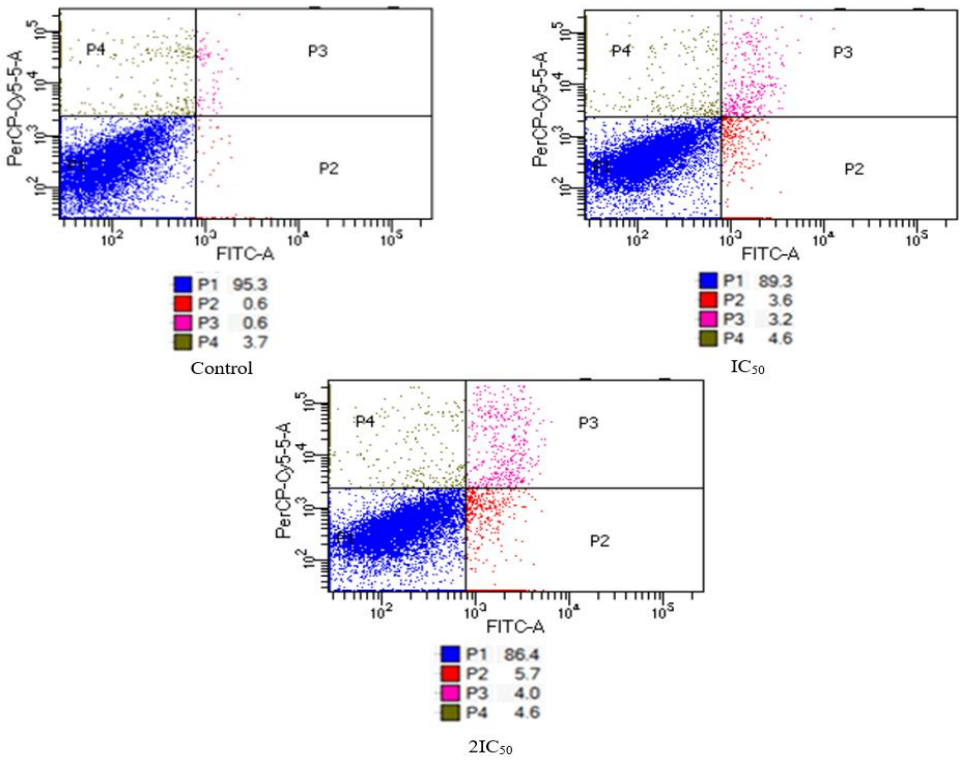


Figure 3. Apoptotic profile of SCR 3 extract against MCF-7 cancer cells. (P1) for viable cells, (P2) early apoptosis, (P3) for late apoptosis and (P4) for necrosis

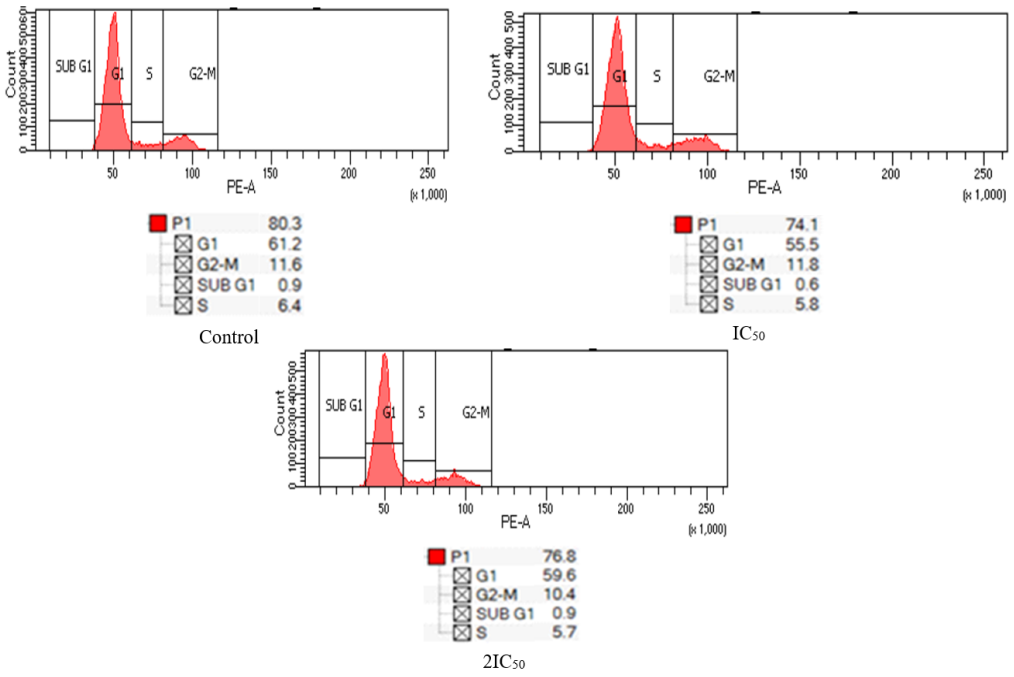


Figure 4. Cell cycle profile of SCR 3 extract against MCF-7 cancer cells

In addition, LC-HRMS of ethyl acetate extract of SCR 3 was used to identify the metabolites quickly with a few samples. The results of biochemical analysis revealed the presence of some substances such as genistein (the primary isoflavone in soybean) that may show a more extraordinary ability to inhibit the proliferation of MCF-7/ER-β1 and MDA-MB-231 cells than parental cells, especially at high concentrations. Additionally, genistein impacts the cell cycle, arresting cells in the G0/G1 phase (Jiang et al. 2018). Moreover, Astuti et al. (2012) studied the effect of genistein and curcumin in modifying cell cycle. They discovered that the effectiveness of treatment depends on the quantity and time of genistein administration. The mortality of T47D cells treated with curcumin was accelerated by genistein, this could be developed as an alternate strategy for treating cancer cells that lack the p53 gene. (+/-)9-HpODE and N1-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-2,2-dichloroacetamide are some additional substances that have detectable antibacterial activity against various bacteria (Sucharitha and Devi 2010; Corteselli et al. 2019). In addition, D-(-)-Mannitol is another substance that exerts a duplex regulatory function by the development of a brown fat-like phenotype and enhancing lipid metabolism. This process causes the browning of 3T3-L1 adipocytes: increasing the expression of genes and proteins specific to brown fat, upregulating lipid metabolism markers, and also increasing the phosphorylation of AMP-activated Protein Kinase (AMPK) and Acetyl-CoA Carboxylase 1 (ACC), indicating a potential part in fat oxidation and lipolysis (Jeon et al. 2021).

The EtOAc extract of SCR 3 (*Aspergillus* sp.) was tested for its ability to modulate cell cycle and apoptosis on MCF-7 cell lines. The results show that extract induces apoptosis (up to 9,7% compared to the control) but did not modulate the cell cycle. From these results, it can be concluded that SCR 3 extract induced apoptosis through a mechanism that did not involve the cell cycle. This study suggests that SCR 3 extract can be a potential source of secondary metabolites that can function as a new source of anticancer agents. Further research is needed to isolate purified compounds from *Aspergillus* sp. and to identify bioactive compounds with the best cytotoxic properties.

ACKNOWLEDGEMENTS

This study was supported by the Indonesian Ministry of Education and Culture (Directorate of Higher Education) through UGM PDUPT Grant no: 1626/UN1/DIT-LIT/DITLIT/PT/2021; 6468/UN1/DIT-LIT/DITLIT/PT/2021; Faculty of Pharmacy, Thesis Supporting Grant 2023.

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