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Cytotoxicity Screening of Endophytic Fungi from *Phaleria macrocarpa* (Scheff) Boerl) Collected in Yogyakarta District

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ABSTRACT

Cancer is a disease with a high prevalence and mortality rate globally. Indonesia's biodiversity has potential to be a source of medicinal ingredients. *Phaleria macrocarpa* (Scheff) Boerl is known to contain active metabolites with potential anticancer properties. Some endophytes are reported to be able to produce secondary metabolites similar to those of their parent plants. This study aimed to obtain endophytic fungi from *P. macrocarpa* that can produce compounds with anticancer properties. Endophytes producing such compounds were explored by screening endophytic fungi producing secondary metabolites from parts *P. macrocarpa* that was collected from the Yogyakarta area. The anticancer bioassay was performed *in vitro* using cancer cells T47D, MCF7, HeLa, and normal Vero cells. Potential extracts were traced for their mechanisms of cell cycle modulation and apoptosis using flow cytometry. Fungal identification was conducted on fungi producing potential cytotoxic extracts. The compound content was then analyzed using LC-HRMS (Liquid Chromatography-High Resolution Mass Spectrometry). Results from the six extracts obtained from fruit, leaf, and stem parts showed that the endophytic extract from the leaves (code PC-L2) had the best cytotoxicity compared to other extracts and demonstrated the best result on MCF-7 cells with an IC_{50} value of 110.66 µg/mL. The produced PC-L2 extract fungi are closely associated with *Clonostachys wenpingii* strain DUCC5606. This extract induced apoptosis, but not through the mechanism of cell cycle modulation.

Keywords: *Phaleria macrocarpa*, cytotoxicity, endophyte fungi, cancer.

INTRODUCTION

Cancer is one of the leading causes of death globally. A total of 70% of cancer-related deaths occur in low- and middle-income countries. If no better treatment of this disease is provided, WHO estimates that cancer-related deaths will increase to 15.3 million by 2040 (Ferlay *et al*., 2021; WHO, 2022). In addition to its high prevalence, patients with cancer also suffers from limitations to therapy, such as high costs, numerous side effects of synthetic drugs in chemotherapy, and development of chemotherapy drug resistance. Side effects of synthetic drugs include hair loss, weight loss due to decreased muscle mass and weakness, and bones becoming potentially porous (Padamsee *et al*., 2017; Barreto *et al*., 2016; Hain, 2020).

Exploration of the anticancer activity of plant endophytes has received considerable attention since the discovery of paclitaxel that was derived from the fungus *Pestalotiopsis microspore.* Found in Himalayan yew; attracting the attention of researchers (Tan & Zou, 2001; Chutulo & Chalannvar, 2018). Endophytes are a source of bioactive substances that can be used to create pharmaceuticals. In addition to being simple to grow, microbes also have comparatively low manufacturing costs. Several endophytes have been reported to be able to produce secondary metabolites similar to their host (Kusari *et al*., 2013). Cheplick and Faeth (2009) stated that endophytic microbes could produce bioactive compounds whose characteristics are similar to or the same as their hosts due to their adaptation to the host, environmental influences, and genetic interactions between the two. For example, camptothecin, produced by the *C. acuminata*, was

isolated from *Fusarium solani,* an endophytic fungus that grows on *C. acuminata* (Pirttilä & Frank, 2011). Two endophytes isolated from *Catharanthus roseus* collection from Bangalore, India, were also reported to produce vinca alkaloids vincristine and vinblastine (Ashoka *et al*., 2017).

Considering the rich biodiversity of Indonesia's natural resources, compounds having anticancer properties can be produced from the endophytes of Indonesian medicinal plants (Lay *et al*., 2023; Astuti *et al*., 2020). Mahkota dewa (*Phaleria macrocarpa*) is a medicinal plant that is widely used and is to have bioactive compounds that have anticancer potential (Tandrasasmita *et al*., 2010; Zhang *et al*., 2012; Shwter *et al*., 2016; Amir *et al*., 2017; Gasong & Tjandrawinata, 2016). However, the cytotoxic compounds from *P. macrocarpa* endophytes are yet to be explored. Endophytic fungi KVM/a and KVM/b isolated from the fruit of *P. macrocarpa* in Jakarta, Indonesia, have cytotoxic activity against MCF-7 cancer cells (Syarmalina *et al*., 2007). Considering the potential of endophytic fungi from *P. macrocarpa*, conducting advanced screening on other endophytic fungi from this plant that grows in another area of Indonesia is necessary to produce secondary metabolites with anticancer properties. This study aimed to find endophytic fungi from *P. macrocarpa* as producers of raw materials for anticancer drugs originating from Indonesian biodiversity.

MATERIALS AND METHODS

Plant Materials and Endophytic Fungi Isolation

P. macrocarpa was obtained from around Universitas Gadjah Mada, Yogyakarta, Indonesia, and then identified in the Plant Taxonomy Laboratory, Faculty of Biology, UGM. Endophytic fungi were isolated from the leaves, stem, and fruit aseptically. Briefly, the plant parts were cleaned with running water for 10 min, sterilized with 70% ethanol for 1 min, and then soaked in 5% sodium hypochlorite for 3 min and 70% ethanol for 30 s before rewashing with sterile distilled water three times. Plant parts were cut into small pieces aseptically according to Lay *et al*. (2023) and then placed in Petri dishes containing PDA media (PDA + chloramphenicol 0.2 mg/mL) and incubated at 25°C.

Endophytic Fungi Fermentation and extraction

Fungal strains isolated from plant parts of *P. macrocarpa* were grown in PDA media at 25°C for seven days. Fermentation was conducted in a 500 mL Erlenmeyer flask containing 200 mL of potato dextrose broth (PDB) media, which had been sterilized by autoclaving (121°C for 20 min) before use and cooled at room temperature. The fermentation medium was inoculated with five plugs (6 mm diameter) of fungal colonies, and the culture was then incubated at room temperature (25°C) with stirring at 120 rpm for 14 days. (Astuti *et al*., 2014). The fermentation broth was filtered using a Whatman filter, and the supernatant was evaporated to one-third of the original volume before ethyl acetate extraction three times.

Cytotoxicity Screening

Cytotoxicity tests were conducted on several cell lines, such as T47D, MCF7, Vero, and HeLa. Cell viability status and level after administration of the test compound were determined by the MTT assay. At the end of the sample treatment in 96-well plates, 100 µL of MTT was added to each well in DMEM medium (final concentration of 0.5 mg/mL). The plate was then incubated for 4 h at 37°C to form formazan. Live cells will react with MTT to form a purple complex. After 4 h, the MTT reaction was stopped by adding 100 µL of 10% SDS stopper reagent to each well and then incubated at room temperature. Absorption was read with an ELISA reader at a wavelength of 570 nm. The absorbance of the sample is used to obtain cell viability based on the following equation:

$$Viability\ (\%) = \frac{Abs.of\ test\ group - Abs.medium}{Abs.cell - abs.medium}\ x\ 100$$

Cell viability data were analyzed using the Microsoft Excel 2019 program to obtain linearity (r) between concentration vs. percentage of cell viability. The sample IC_{50} value was then calculated. The IC_{50} value of each sample obtained from the treatment on each cancer cell line (MCF-7, T47D, HeLa) and the Vero cell line was further used for determining the SI value. The formula for the SI value:

$$Selectivity\ incex = \frac{IC_{50}\ sample\ against\ Vero\ cells}{IC_{50}\ sample\ against\ cancer\ cell\ lines}$$

A compound is more selective in killing cancer cells than normal Vero cells if it has an SI value > 3 (Bézivin *et al*., 2003).

Apoptosis test with Annexin V-Flow cytometry

The apoptotic test was conducted on extracts with cytotoxic capability. A total of 5×10^3 cells/well

were pipetted to 6-well plates for 24 h and then added with the extract (incubated in a CO_2 incubator for 24 h). Cells were harvested, washed with PBS, resuspended with binding buffer, and treated with Annexin V-FITC reagent for 10 min. The cells were then analyzed using flow cytometry to obtain a histogram of the distribution of live cells, early apoptosis, late apoptosis, and necrosis.

Cell cycle assay with PI-Staining Flow cytometry

This test was conducted on extracts with cytotoxic capability. A total of 5×10^3 cells/well were distributed into 6-well plates for 24 h. The cells were then given several extract concentrations (incubated for 24 h). The cells were washed with PBS and then treated with a trypsin solution (incubated for 2 min). Afterward, the cells were treated with PI reagent (incubated for 10 min) and analyzed using a flow cytometer to determine the cell distribution and proportions of each cycle.

Identification Endophytic Fungi of *P. macrocarpa*

Fungi dentification was conducted on the fungi with the most potent activity based on the initial screening results of the culture extract. Identification was performed to obtain the identity or type of fungus with anticancer potential. This identification was conducted molecularly (DNA isolation, PCR sequencing, and phylogenetic tree by the NCBI blast tree method). Data analysis was performed with MEGA 6.0 software.

Analysis of Compounds within the Bioactive Extracts

Extracted samples with potential cytotoxic activity from endophytic fungi of *P. macrocarpa* (PC-L2) were analyzed for the content of compounds using LC-HRMS at the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, Indonesia.

Data analysis

Each piece of data is presented as mean ± SD. The statistical analysis relies on normality values evaluated using one-way analysis of variance or the Kruskal–Wallis test (SPSS version 16 program). $P < 0.05$ was considered significant with a 95% confidence interval.

RESULTS AND DISCUSSION

The fungal strain was isolated from the stem, leaves, and fruit *of P. macrocarpa*. Six types of endophytic fungal strains were obtained: three from the fruit (PC-F1, PC-F2, and PC-F3), two from the leaves (PC-L1 and PC-L2), and one from the stem (PC-S1). The tips of the white fungal hyphae from the edge of the segments on the plate containing potato dextrose agar (PDA) were visible in the form of cottony and fibrous colonies with various colors and morphologies (Figure 1). Cultures on PDA plates were fermented on PDB for 14 days before using ethyl acetate for extraction. The solvent was then evaporated to obtain an ethyl acetate extract ready for cytotoxicity screening.

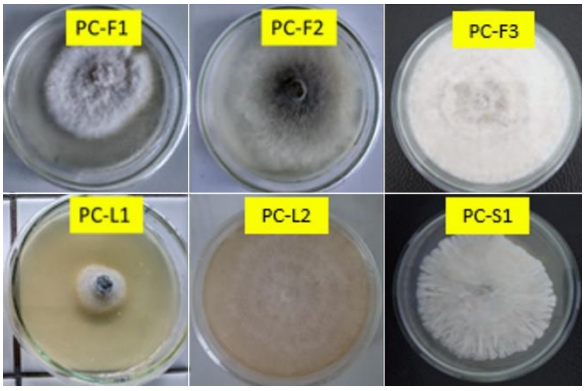

Figure 1. Endophytes isolated from leaves, stem, and fruit of *P. macrocarpa* on PDA.

Cytotoxicity

Cytotoxic activity tests on a series of cancer cell lines were conducted *in vitro* to determine the potential of ethyl acetate extract of the endophytic fungus *P. macrocarpa.* Three cell lines were used as the cancer cell model (MCF-7, T47D, and Hela cell), and the Vero normal cell model was utilized. The cytotoxic test was performed on each endophytic fungi ethyl acetate extract at various concentration series (15.265, 31.25, 62.5, 125, and 250 µg/mL [data not shown]). The extract with the highest potential as a cytotoxic agent in v*itro* is PC-L2 against MCF-7 cells (Table I).

PC-F1 and PC-F2 extracts destroyed MCF-7 cells, reaching a maximum viability of 55.51% and 51.49%, respectively, at a concentration of 250 µg/mL. PC-L2 extract can destroy more than 50% of cell growth, with a maximum viability of 45.37%. The IC_{50} value in the PC-L2 extract was 110.66 µg/mL with a selectivity index of 11.16. A compound is more selective in destroying cancer cells than normal Vero cells if it has an SI value > 3 (Bézivin *et al*., 2003). Cytotoxic activity screening using other types of cell lines demonstrated inferior results (Table I).

Table I. Cytotoxicity of EtOAc extract against HeLa, MCF-7, T47D, and Vero cells

Cell Lines	Edophytic Fungal Cultures Extracts % viability					
	PC-F1 (250 µg/mL)	PC-F2 (250 µg/mL)	PC-F3 (250 µg/mL)	PC-L1 (250 µg/mL)	PC-L2 (250 µg/mL)	PC-S1 (250 µg/mL)
Hella	88.10 ± 0.03	84.53 ± 0.008	76.67 ± 0.31	80.4 ± 0.01	58.61 ± 0.02	80.78 ± 0.2
MCF-7	55.51 ± 0.004	51.49 ± 0.02	107.29 ± 0.04	103.42 ± 0.02	45.37 ± 0.02	110.66 ± 0.03
T47D	75.22 ± 0.01	78.89 ± 0.08	70.30 ± 0.03	92.95 ± 0.08	79.62 ± 0.06	71.27 ± 0.14
Vero	75.48 ± 0.05	64.14 ± 0.009	91.32 ± 0.06	94.64 ± 0.003	74.74 ± 0.007	80.63 ± 0.02

PC-F1: *P. macrocarpa*-fruit1; PC-F2: *P. macrocarpa*-fruit2; PC-F3: *P. macrocarpa*-fruit3, PC-L1: *P. macrocarpa*-leaves1; PC-L2: *P. macrocarpa*-leaves2; PC-S1: *P. macrocarpa*-stem1.

Table II. Distribution of apoptotic and necrotic cells after administration of PC-L2 extract and Cell cycle profile after administration of PC-L2 extract

Treatment	Cell Distribution (%)							
	P1	P2	P3	P4	G_1	G_2/M	Sub G_1	S
Control	95.3	0.6	0.6	3.7	76.2	14.4	1.1	7.9
2xIC_{50}	79	5.0	10.5	5.7	78.3	14.2	1.1	6.0
IC_{50}	84.6	5.8	6.6	3.6	77.0	14.8	0.9	7.1

P1: Viable Cells; P2: Early Apoptosis; P3: Late Apoptosis; P4: Necrosis

The categories of cytotoxic activity against cancer cells can be classified as follows: very active if the IC_{50} value is <10 µg/mL; active if the IC_{50} value is 10–100 µg/mL; quite active if the IC_{50} is 100–500 µg/mL; and less active if the IC_{50} value is >500 µg /mL (Gusungi *et al*., 2020). The PC-L2 extract was quite active in inhibiting the growth of MCF-7 cells based on this classification. This study shows that the PC-L2 extract was analyzed for its apoptotic capability and mechanism of cell cycle modulation.

PC-L2 Extract Induces Apoptosis

The distribution percentage of MCF-7 cells due to the administration of PC-L2 extract at concentrations of IC_{50} (110.66 µg/mL) and 2xIC_{50} (221.32 µg/mL) was different from the control (Figure 2A). While the percentage of living cells in control was nearly 100% with almost minimal change toward apoptosis or necrosis, the administration of PC-L2 extract with concentrations of IC_{50} or 2xIC_{50} showed an increase in the percentage of cells that underwent apoptosis or necrosis (Table II).

PC-L2 Extract on MCF-7 Cell Cycle Modulation

Cell cycle analysis by flow cytometry was performed to determine the distribution of cells in each phase of the cell cycle. Administration of PC-L2 extract did not differ significantly from control cells in the cell cycle profile (Figure 2B; Table II). The results suggested that the PC-L2 extract induced apoptosis through a mechanism other than cell cycle modulation.

Identification of Endophytic Fungus PCL2

The identification results of the endophytic fungus PC-L2 isolated from the leaves of *P. macrocarpa* demonstrated its similarity to the species *Clonostachys wenpingii* strain DUCC5606 (Figure 3). This identification was performed molecularly through DNA isolation, PCR sequencing, and phylogenetic tree by NCBI. This closeness is based on the BLAST Results Hit Against the NCBI Database, which revealed a percentage identity level of 99.99%. *Clonostacys* is distributed worldwide and produces various secondary metabolites with numerous biological activities (Han *et al*., 2020). However, no studies report the secondary metabolites or the biological activity of *Clonostachys wenpingi.*

The secondary metabolites of approximately 18 out of 65 species in the genus Clonostachys have currently been studied. The secondary metabolite class of *Clonostachys* mainly contains nitrogenous compounds, polyketides, and terpenoids (Zeng and Zhuang, 2022; Han *et al*., 2020).

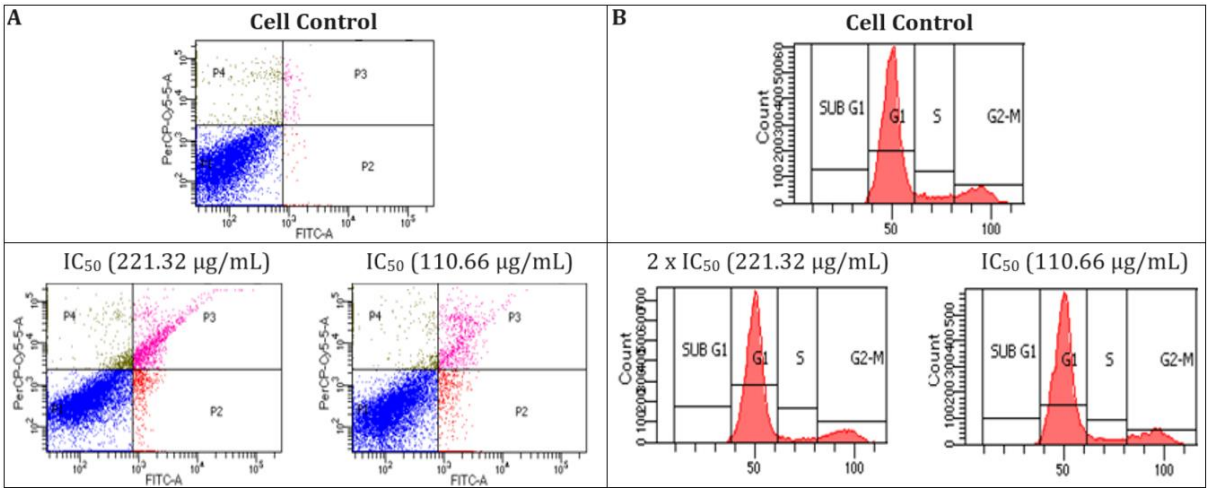

Figure 2. (A) Effect of PC-L2 extract on the apoptotic profile of MCF-7 cells. (B) Histogram profile of PC-L2 extract on MCF-7 cell cycle. Abbreviations: P2, viable cells; P3, early apoptosis; and P4, late apoptosis; P5, necrosis.

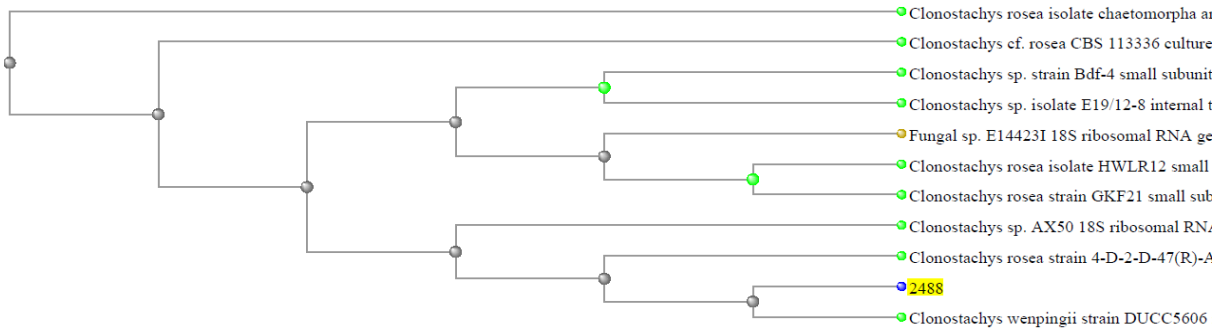

Figure 3. Phylogenetic tree analysis of the endophytic fungus PC-L2

Clodepsipeptide compounds from *Clonoctachys sp.,* Cyclo-(Gly-D-Leu-D-allo-Ile-LVal-L-Val-D-Trp-β-Ala) from *Clonostachys rosea*, and IB-01212 from *Clonostachys sp.* ESNA-A009 were reported to have anticancer activities (Samuel and Prabakaran, 2011; Abdel-Wahab *et al*., 2019; Li *et al*., 2020).

Metabolites of PC-L2 Extract

Several secondary metabolites within the PC-L2 extract based on LC-HRMS analysis (Figure 4). The PC-L2 extract has the following 15 top compounds: Perfluorooctanoic acid (1), Dodecyl sulfate (2), 4-Dodecylbenzenesulfonic acid (3), Myristyl sulfate (4), (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid (5), 5-[({(2R,4S,5R)-5-[1-Methyl-3-(2-thienyl)-1H-pyrazol-5-yl]-1-azabicyclo[2.2.2]oct-2-yl}methyl)amino]-5-oxo pentanoic acid (6), NP-016455 (7), 5,7-Dihydroxy-4-methyl coumarin (8), D-(-)-Quinic acid (9), NP-016455 (10), Perfluorohexanoic acid (11), 2,2'-Methylenebis(4-methyl-6-tert-butylphenol) (12), (2E)-4-Hydroxy-4-{4-hydroxy-2-[(1E)-6-hydroxy-1-hepten-1-yl]cyclopentyl}-2-butenoic acid (13), 5α-Dihydrotestosterone glucuronide (14), and NP-018716 (15).

Some of the compounds, such as (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid (Lesmana *et al*., 2021), 5,7-Dihydroxy-4-methylcoumarin (Zheng *et al*., 2020), and 2,2'-Methylenebis(4-methyl-6-tert-butylphenol), have been reported to have antioxidant activity (Tanaka *et al*., 1990). In addition, D-(-)-quinic acid has been reported to have potential as an NFκB inhibitor. D-(-)-quinic can prevent free radical oxidation and inhibit the release of inflammatory mediators such as TNFα, NFκB, and NO (nitric oxide). These mediators are involved in inflammation and cellular stress (Liu *et al*., 2020; Pero *et al*., 2009).

Inflammation is often associated with the development of cancer. Moreover, inflammation can be intrinsically elicited by cancers due to mutations and can contribute to the development of malignant cancers through the recruitment and activation of inflammatory cells (Todoric *et al*., 2016; Singh *et al*., 2019).

The capability of the PC-L2 extract to inhibit the development of MCF-7 cancer cells can be attributed to the presence of compounds with anticancer properties according to several studies. However, further examination is necessary to prove this finding.

CONCLUSION

In this study, six endophytic fungi were isolated from *P. macrocarpa*. An endophytic fungus having anticancer properties in its leaves was identified as *Clonostachys wenpingii* strain DUCC5606. The ethyl acetate extract of PC-L2 demonstrated relatively active cytotoxic activity, with an IC_{50} of 110.66 µg/mL against the MCF-7 cell line. The PC-L2 extract did not modulate the cell cycle and may induce apoptosis via other mechanisms.

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