

ANTICANCER ACTIVITIES OF SECONDARY METABOLITES PRODUCED BY TAXUS ENDOPHYTIC FUNGUS PHOMOPSIS CHIMONANTHI

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ABSTRACT

Secondary metabolite by the *Taxus* endophytic fungus strain TsC3 culture obtained from the West Java Forest has been produced. Production of secondary metabolite had done by aerobic fermentation for 7 days, 30°C, 120 rpm in PDB (potato dextrose broth) media. The broth of fermentation was extracted by ethyl acetate. Various concentrations of the ethyl acetate extract in (0- 800) mcg/mL were prepared and test against T47D cancer cell line. Cytotoxic test has be done by MTT method. IC₅₀ value of ethyl acetate extract was 3 mcg/mL. The result of fungus identification by morphology and molecular 18S rRNA was *Phomopsis chimonanthi*

Key words: *Phomopsis chimonanthi*, fermentation, isolation, cytotoxicity, identification, *taxus*

ABSTRAK

Telah diproduksi metabolite sekunder oleh fungi endofitik *Taxus* strain TsC3 yang diperoleh dari hutan di Jawa Barat. Produksi metabolit sekunder dilakukan secara fermentasi aerobik selama 7 hari, 30°C, 120 rpm dalam media PDB (potato dextrose broth). Cairan hasil fermentasi diekstrak dengan etil asetat. Sejumlah 0-800 mcg/mL dari variasi konsentrasi ekstrak etil asetat diuji terhadap sel kanker T47D. Uji sitotoksik dilakukan dengan metode MTT. Nilai IC₅₀ dari ekstrak etil asetat adalah 3 mcg/mL. Hasil identifikasi fungi secara morphology dan molekular 18S rRNA adalah *Phomopsis chimonanthi*

Kata kunci: *Phomopsis chimonanthi*, fermentasi, isolasi, sitotoksitas, identifikasi, *taxus*

INTRODUCTION

Taxus sp. is a medicinal plant that has produced anticancer drug and proved clinically. This rare plant can grow and produce its active compound only in its suitable environment. Isolation of endophytic microorganism from the part of its body and produce in the large scale by fermentation is an effective method for inducing the secondary metabolite anticancer together with to take care of its everlastingness in their habitats. Therefore, studies on the endophytic fungi of those medicinal plants will be great value to ecology and pharmacology. *T. mairei*, one major source of taxol as a impotent antitumor medicine⁽¹⁾.

The endophytic fungus *Phomopsis sp.* is a rich source of biologically active secondary metabolites⁽²⁾ and fungi are known to produce a vast array of secondary metabolites that are gaining importance for their biotechnological applications. The reports on secondary metabolites from the antagonists to inhibit pathogens are also studied by several scientists⁽³⁾. Chemical investigation of the ethyl acetate extract from the culture broth and the cells of the endophytic fungus *Phomopsis sp.* PSU-D15 led to isolation three new compounds, namely phomoenamamide, phomonitroester and deacetylphomoxanthone B⁽⁴⁾.

T47D cell was isolated from human breast cancer. In laboratory, cell grows by sticking in the base of flask. T47D cell has expressed

characteristic estrogen receptor +. Molecularly, it had missense mutated at 194 residue (in zinc-binding domain L2) *p53* gene. The mutation has caused *p53* protein can not tie with response element at DNA so that the *p53* protein lose its function. If *p53* can not tie with response element at DNA then its ability to regulate the cycle of cell and the effect of apoptosis of cell was decreased ⁽⁵⁾.

In this study we have tested the anticancer properties of ethyl acetate extract of *Phomopsis c.* against T47D cancer cell line.

MATERIALS AND METHOD

Taxus endophytic fungus strain TsC3 culture

Taxus endophytic fungus strain TsC3 culture had isolated from the inner bark and leaf of *Taxus sumatrana* in cornmeal agar, malt extract, yeast extract media ^(6,7).

Identification of active strain TsC3 of *Taxus* endophytic fungus

Morphology identification

Identify of morphology of fungus based on fenotip characteristic on microscopic observation by lactic acid or trypan blue solution application ^(8,9).

Molecular 18S rRNA identification

Sequences 18S rRNA has been done by incubation of endophytic microorganism for 72 hours in potato dextrose broth media, extraction of biomass of fungi to find the DNA by reagent nucleon phytopure (Amersham Life Science), PCR amplification at D1/D2 region by NL-1 Primer 5'—GCA TAT CAA TAA GCG GAG GAAAAG-3' and NL 4 Primer 5'—GGT CCG TGT TTC AAG ACG---3', PCR amplification in ITS by ITS 4 Primer 5'—TCC TCC GCT TAT TGA TAT GC-3' and ITS 5 Primer 5'—GGA AGT AAA AGT CGT AAC AAG G --3', purification of PCR product by PEG precipitation method and continuing with sequencing cycles, purification of the result of sequencing cycles by ethanol purification method, analysis of nitrogen base sequence reading by automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems), trimming the sequence result of raw data by MEGA 4 program and assembling with BioEdit program and then convert in FASTA format ^(10,11,12).

Production and isolation of anticancer substances from endophytic fungi

Production of secondary metabolite had done by fermentation of strain TsC3 culture in 0.5 L volume of flask for 7 days, 30°C, 120 rpm in PDB (potato dextrose broth) media and isolation of anticancer substances by extraction of the broth of fermentation by ethyl acetate organic solution ⁽¹³⁾.

Bioassay of anticancer against T47D breast cancer cell line

Cytotoxic test by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method has done for bioassay anticancer against T47D breast cancer cell line (was obtained from the faculty of Pharmacy, Gajah Mada University (UGM), Yogyakarta, Indonesia) by prepared the solution test in (0-800) mcg/mL of ethyl acetate extract of strain culture of *Taxus* endophytic fungus dissolved in DMSO. Propagation and harvest of T47D cell from liquid nitrogen tank by prepared the cell in water bath 37°C and ethanol 70%. Cell removed to a conical tube aseptically that contain RPMI 1640 (Rosewell Park Memorial Institute) medium (Gibco). Centrifuges the suspension in 10.000 rpm for 5 minutes, discard the supernatant and replace with new RPMI medium in suspension. Centrifuge the suspension in 10.000 rpm for 5 minutes. The supernatant discarded and add 1 mL grow medium that contain 20% PBS to biomass and made the homogeneous suspension. The cell grew at several tissue culture flasks in 37°C and flow of 5% CO₂ incubator. The medium changed after 24 hours and grew the cell until confluent and enough for research. After the cell enough, replaced the medium with new RPMI 1640 medium five times and moved cell from the wall of flask. Cell moved to sterile conical tube and add RPMI medium until 10 mL volume and centrifuged in 10.000 rpm for 5 minutes. Cell washed by the same medium and counted the number of cell by hemositometer. Suspension cell was made in 2 x 10⁴ sel/100 L. Cell was distributed to 96 well plates then incubated for 48 hours in CO₂ incubator. Continued with added various concentrations of test solution then incubated for 48 hours. At the last incubation, MTT in PBS (sigma) added to each well. The incubation was continued for 6 hours in 37°C until the formazan could be formed. Alive cell will convert MTT to purple formazan. MTT reaction was stopped by stopper reagent then

incubates overnight in room temperature. Read the absorption by ELISA reader in λ 550 nm then converted the value of absorbance in viable percentage⁽¹⁴⁾.

RESULTS AND DISCUSSION

Cytotoxic test of strain TsC3 culture of *Taxus endophytic fungus* by the MTT method

Cytotoxic test had be done for determining the concentration of strain TsC3 culture to combat the cell growth until 50% (IC₅₀) by incubate T47D cell in serial concentrations of ethyl acetate extract of strain TsC3 culture for 48h. Incubation was finished by reagent MTT. MTT is tetrazolium bromida. It metabolized the system of enzyme suksinat dehidrogenase that changed to purple formazan. Intensity of color of formazan was measured by Elisa reader. Intensity of formazan color was proportional the number of alive cell.

Table 1. Cytotoxic test of ethyl acetate extract of strain TsC3 culture against T47D cell in (20 – 800) mcg/mL for 48h incubation.

Extract concentrations (mcg/mL)	Viable cells (%) rep 1	Viable cells (%) rep 2	Viable cells (%) rep 3	Average of Viable cells	Standard of deviation
22	8.56	11.0	8.11	9.24	1.57
45	3.62	5.87	6.99	5.50	1.71
90	6.77	7.22	8.79	7.59	1.06
181	2.73	4.07	2.73	3.18	0.77
363	5.42	12.16	8.56	8.71	3.36
726	9.91	7.67	10.58	9.39	1.52

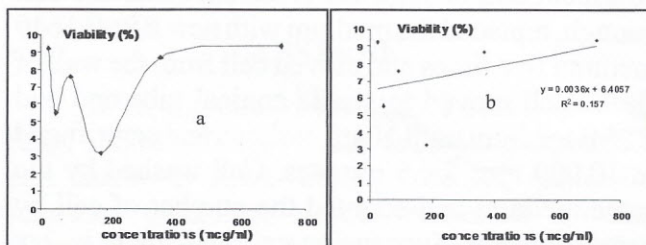


Figure 1 . Percentage of resistance of T47D growth by treatment with ethyl acetate extract of strain TsC3 culture in (20-800) mcg/mL for 48h incubation (a) and its linear (b).

The potency of cytotoxic effect of the extract will show in IC₅₀'s values. The higher the value of IC₅₀, the lower the potency of cytotoxicity. Ethyl acetate extract of strain TsC3 culture has cytotoxic potency whereas 22 mcg/mL has caused death of

more than 90% cell (Table 1 and Figure 1).

Table 2. Cytotoxic test of ethyl acetate extract of strain TsC3 culture against T47D cell in (0 - 11) mcg/mL for 48h incubation

Extract concentrations (mcg/mL)	Viable cells (%) rep 1	Viable cells (%) rep 2	Viable cells (%) rep 3	Average of Viable cells	Standard of deviation
11	19.30	15.79	7.93	13.34	4.68
6	28.53	28.15	43.21	33.30	7.80
3	50.93	43.98	35.36	43.42	16.89
1.5	79.00	105.91	74.75	86.55	24.91
0.8	133.07	150.71	101.53	92.09	6.61
0.1	98.18	85.05	93.03	92.09	6.61

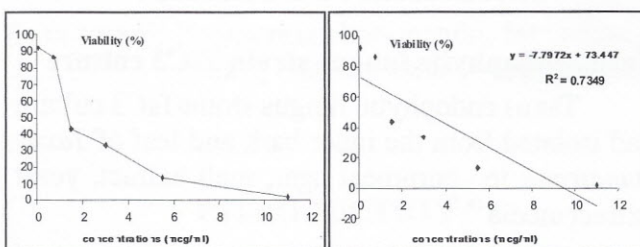


Figure 2. Percentage of resistance of T47D growth by treatment with ethyl acetate extract of strain TsC3 culture in (0-11) mcg/mL for 48h incubation (a) and its linear (b).

For interpreting of cytotoxic effect of extract, its concentration must be decreased to the smallest concentration. The linearity at Figure 2 and Table 2 showed the relation of extract concentrations against alive cell, TsC3 had IC₅₀: 3 µg/ml.

Morphology of T47D cell by or without treatment with ethyl acetate extract of strain TsC3 culture can show in Figure 3.

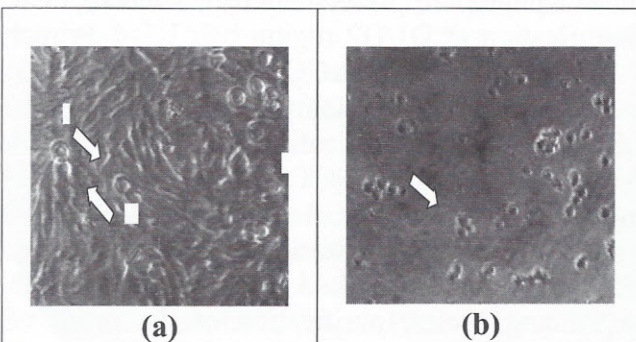


figure 3. Morphology of T47D cell for 48h incubation (a) cell without treatment (b) cell with treatment by 22 mcg/mL ethyl acetate extract of strain TsC3 culture, i. Alive cell, like a piece of leaf that stick on the base of well and ii. Cells have already changed the morphology, look to become round and float.

Ethyl acetate extract of strain TsC3 culture has been potential as cytotoxic agent; whereas it has combated the growth of T47D cell (it was very resistant for apoptosis). The effect of blocking cover in two lines that was combating proliferation of the cell by modulation of cell cycle program or combating the transduction signal and spurring of apoptosis.

Identification of active *Taxus* endophytic fungus strain TsC3 culture by morphology and molecular 18S rRNA

Morphology of *Taxus endophytic fungus* by microscope in PDA media was likes cotton in brown white and grey color. The hypa consists of long, slender and branched filaments in transparent/hyaline and brown/dematiaceous color. It can not find structure in clamp connection.

Molecular identification of Taxus endophytic fungus by BLAST The result of DNA sequencing in

FASTA format to looking for the online homology in DNA data base center in DDBJ (<http://www.ddbj.nig.ac.jp>) or NCBI (<http://www.ncbi.nlm.nih.gov/>) relationship analysis and production of phylogenetic tree by Clustal X program and NJ plot was *Phomopsis chimonanthi* (see figure 4).

CONCLUSIONS

Ethyl acetate extract of *P. chimonanthi* can produce anticancer substances and very potential to be the source of anticancer agents ($IC_{50} = 3$ mcg/mL).

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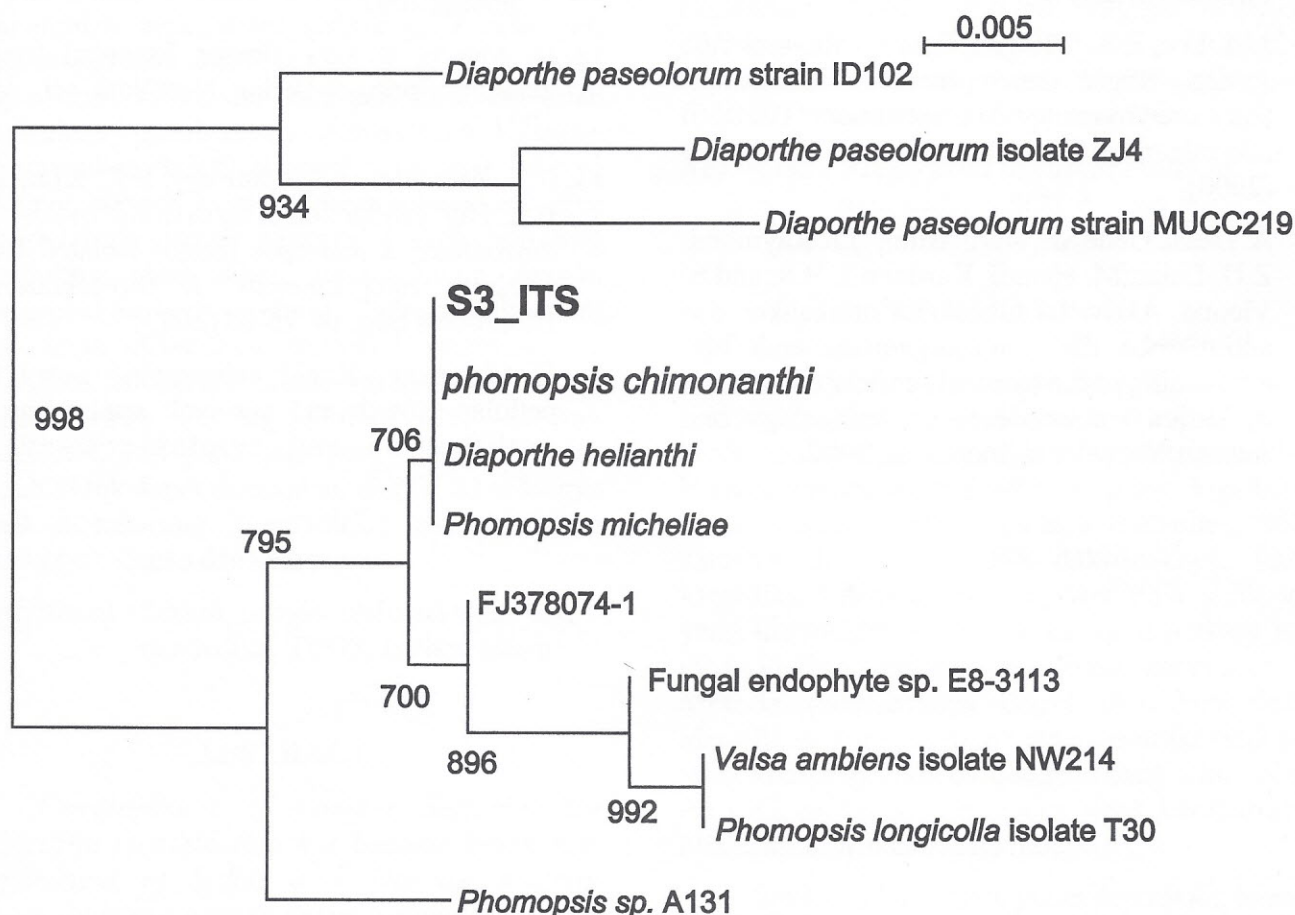


Figure 4. Phylogenetic tree of *Phomopsis chimonanthi*

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