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Identification, Antimicrobial Activities and Cytotoxicity of *Talaromyces purpurogenus* T013

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ABSTRACT

In the study, Talaromyces purpurogenus T013, a filamentous fungi belonging to Talaromyces which was identified had been isolated from the high value Taxus baccata subsp. wallichiana. By phylogeny analysis, T. purpurogenus T013 showed the close to other T. purpurogenus and Penicillium prupurogeum. In order to find out a new source of antimicrobial and anticancer agents, T. purpurogenus cultured in YM was against five different kinds of pathogens including Salmonella typhi (16.77 ± 0.21 mm), Pseudomonas aeruginosa (16.17 ± 0.21 mm), Serratia marcescens (15.50 ± 0.21 mm), Listeria monocytogenes (16.37 ± 0.15 mm) and Vibrio parahaemolyticus (15.43 ± 0.21 mm) by using the agar well diffusion method. There was cytotoxic ability on Hela cancer cell line at the concentration (100 mg/mL) by using sulforhodamin B assay. The chloroform fraction showed antimicrobial activities that suggested the purified agent with antimicrobial in this fraction will be obtained so far.

Keywords: Identification; Agar well diffusion method; Cytotoxicity; Antimicrobial activities

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INTRODUCTION

Taxus baccata subsp. wallichiana is a source of the chemical precursors to the anticancer drug paclitaxel (Taxol) and other antimicrobial agents. However, the yield of these biological compounds in *Taxus baccata subsp. wallichiana* is very limited. Scientists has found out that endophytic fungi isolated from *Taxus baccata subsp. wallichiana* also have the ability to produce biological compounds such as *Pestalotiopsis microspora* strain Ne-32 and strain No.1040 [1].

Talaromyces purpurogenus (*T. purpurogenus*) CBS 28636 was isolated and characterized by dark grey-green colonies with mycelium varying from pinkish to yellow and yellow red, as well as the production of red pigments on potato agar. *T. purpurogenus* is claimed to produce rubratoxins [2-4]. *T. purpurogenus* has also been regarded as a producer of glauconic acid rather than rubratoxins. Rubratoxin B is mutagenic, hepatotoxic, nephrotoxic and splenotoxic to several animals [5-9]. The first human rubratoxicosis was reported by Richer et al. [10]. However, it has potential as an anti-tumor agent [11,12]. *T. purpurogenus* hasn't been detected the antimicrobial activities.

Nowadays, antimicrobial resistance is an increasingly serious threat to global public in which bacteria, parasites, viruses and fungi have the ability to resist to antibiotics, antifungals, antivirals, etc. As a results, the drugs become ineffective and infections persist in the body, leading to the risk of spread to others. *Salmonella typhi*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Listeria monocytogenes* and *Vibrio parahaemolyticus* are several bacteria that had been found to have the ability to resist to antimicrobial drugs [13-16]. Moreover, *T. purpurogenus* was usually isolated outdoor.

For that reason, this study tried to detect the biological activities of *T. purpurogenus* isolated from *Taxus baccata subsp. wallichiana* in order to find out a new source of antimicrobial and anticancer agents production that serve for resistance in the future. *T. purpurogenus* is expected to have the antimicrobial activities on different pathogens such as *Salmonella typhi*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Listeria monocytogenes* and *Vibrio*

parahaemolyticus. The purification step using polar solvent and non-polar solvent is able to collect the pure substance that has the antimicrobial ability with above pathogens and cytotoxicity on Hela cells [17,18].

EXPERIMENTAL SECTION

Fungi Collection and Screening

The trunk, leaves were separated and cultured in PDB (Potato Dextro broth) for enrichment. After 4-5 days, the culture was spread onto PDA containing *Bacillus subtilis* as indicator. The colonies inhibited *Bacillus subtilis* were transferred on YM medium containing 1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 2% agar which is used for maintenance and sporulation of fungi.

Identification by 28S sRNA Sequence Analysis

RNA was isolated and checked on 1% agarose gel universal primers; (F-5'-GGGTGACCAACGCCCGCAGGCCCCT-3' and R-5'-GGAAGTAAAAGTCGTAACAAGGT-3') with initial denaturation at 95°C for 5 mins followed by 35 cycles of 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 3 mins. Then, the reaction mixture was kept at 72°C for 10 mins, cooled to 4°C. Polymerase chain reaction product was purified by gel elution and sequenced. The nucleotide sequence was analyzed using Blast search. The software Molecular Evolutionary (MEGA6) and Blast web were used to construct the phylogeny tree neighbor joining treeing algorithm for identified *Talaromyces*.

Antimicrobial Test

Pathogens used for antimicrobial test were *Salmonella typhi* ATCC 19430 (P2), *Pseudomonas aeruginosa* ATCC 27853 (P3), *Serratia marcescens* ATCC13880 (P6), *Listeria monocytogenes* [17] and *Vibrio parahaemolyticus* (VSA). All these pathogens were cultured in Luria-Bertani (LB) broth and gram-stained to check the purity. Before the test, the pathogen samples were measured the absorbance to determine the optical density (OD). The test was performed on LB agar plate [19]. The supernatant extracted from *T. purpurogenus* culture will be used as the sample of the test.

The antimicrobial test was performed using agar well diffusion method [20]. M1 medium was used as the negative control for the test while tetracycline was used as the positive control. Applying aseptic techniques, using a small sterile metal stick to punch into the LB agar plate to form wells. Amount (10^6 CFU/mL) of pathogen cultures (*Salmonella typhi*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Listeria monocytogenes* and *Vibrio parahaemolyticus*) was spread onto the surface of the plate, using sterile swabs. Finally, the supernatant was pipetted into the wells. All the plates were incubated at room temperature. After ten to twelve hours, the inhibition zones formed on the wells were measured and analyzed.

Cytotoxicity Test

Sulforhodamine B and different cancer cell lines (HepG2, MCF-7, Hela and NCI-H460) were used for the test. The sample prepared for this test was the dry powder extracted from *T. purpurogenus* culture mixed with EMEM/10% FBS medium which was used for culture of cancer cell lines. Positive control of the test was the mixture of cells and camptothecin at the concentration 0.07 µg/ml. The mixture of cells and the solvent that dissoved the *T. purpurogenus* dry powder (EMEM/10% FBS culture medium) was used as negative control. The test for the sample at each concentration was designed with four wells. Two wells contained the cancer cell line, the culture (EMEM/ 10% FBS) and the tested substance (*T. purpurogenus* dry powder), the other two contained only the culture and the sample (blank). The cytotoxicity test was performed by sulforhodamine B assay [21]. It is a simple colorimetric method for determining sensitivity and cytotoxicity of a substance. Sulforhodamine B (SRB), a negatively charged dye which can bind electrostatically with the positively charged parts of proteins will be the main reagent for the assay. The amount of binding dye will reflect the total cellular protein.

Firstly, the cancer cell line preserved in liquid nitrogen will be thawed and cultured until the fourth generation (P4). Next, the cells will be cultured in culture flasks to achieve the coverage of about 70-80%. The cells now is covered into wells of 96-well plate at a density of 10^4 cells per well (In the case of HepG2, MCF-7 and Hela) or 7.5 x 10^3 cells per well (In the case of NCI-H460 cancer cell line). After that, incubate the cell at 37^oC in 5% CO_2 for 24 hours. After incubation, the wells are supplied with the medium of the tested substance with the double concentration. Continue to incubate the cells at 37^oC in 5% CO_2 for 48 hours. The cells in the wells now are fixed with trichloroacetic acid (TCA).

Secondly, cells were dyed with SRB: 0.2% SRB was loaded into each well and incubated at room temperature (25^oC) for 5 to 20 minutes. After that, SRB was discarded and the cells were washed gently with 1% acetic acid for 5 times. Let the cells dry at room temperature (25^oC) from 12 hours to 24 hours. The results were obtained by

loading 200 µL Tris-base into each well, then shaking for 10 to 15 minutes until the SRB was dissolved completely and measuring the optical density value at wavelength 492 nm and 620 nm.
The cytotoxic percentage was calculated by the formula:

$$\%I = \left(1 - \frac{OD_{sample}}{OD_{control}}\right) \times 100\%$$

Notes:

OD_{Cell}: OD value of well containing cells
OD_{Blank}: OD value of blank well
OD_{sample}: OD value of sample obtained from formula (1) and (2)
$OD_{control}$: OD value of control obtained from formula (1) and (2)

Preliminary Purification

Supernatant extracted from *T. purpurogenus* culture after 240h of culturing will be used for purification. To purify the supernatant, chloroform and petroleum ether were used as non-polar solvents and methanol as polar solvent. The supernatant was added into three sterile falcons. Petroleum ether was used to extract the non-polar compounds with the alternative ratios (petroleum ether: extract = 2:1; 1:1; 1:2). The mixtures were mixed vigorously. After collection of the petroleum ether fractions, the remaining extract was alternatively added with chloroform with the suitable ratio of chloroform: supernatant (2:1; 1:1; 1:2). Repeating the same steps as petroleum ether, the mixtures were mixed vigorously. The collected chloroform fractions were evaporated and then the powder was dissolved in distilled water for antimicrobial activity and cytotoxicity. The remaining extract continued to be purified with methanol and applied the same process. The fraction was used for antimicrobial activity test.

RESULTS AND DISCUSSION

Phylogeny Tree Analysis

Using Blast search, isolated fungi had 28 SrRNA showing 99% homology to *Talaromyces purpurogenus* IAM15392. *T purpurogenus* was classified formerly in *Penicillaceae* because of their close root. However, *T. purpurogenus* was classified in different category nowadays. The isolated fungi lie within the tree of *T. purpurogenus*. The isolated fungi were identified as *Talaromyces purpurogenus* AD1 (Figure 1).

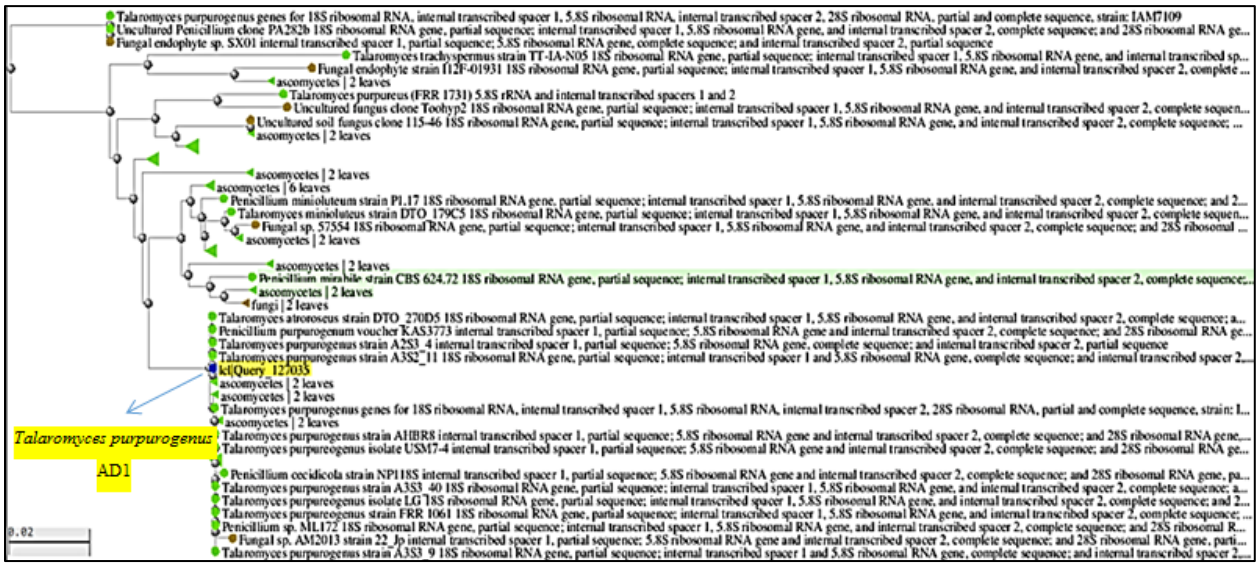

Figure 1: Phylogeny tree of isolated microorganism using neighbor-joining

Antimicrobial Activities

T. purpurogenus expressed antimicrobial activity to all strains of tested pathogens. Results of antimicrobial test were recorded in Table 1.

Table 1: Diameter of inhibition zones resulted from antimicrobial test of *T.purpurogenus* extract with different strains of pathogen

Time	*Salmonella typhi (P2)*	*Pseudomonas aeruginosa (P3)*	*Serratia marcescens (P6)*	*Listeria monocytogenes (17)*	*Vibrio parahaemolyticus (VSA)*
24h	8.07 ± 0.15	7.23 ± 0.25	6.87 ± 0.32	8.43 ± 0.15	5.23 ± 0.25
48h	9.33 ± 0.31	8.77 ± 0.25	8.50 ± 0.26	9.60 ± 0.26	7.13 ± 0.32
72h	10.73 ± 0.31	12.17 ± 0.21	10.23 ± 0.15	12.07 ± 0.49	11.00 ± 0.26
96h	12.23 ± 0.21	13.30 ± 0.36	11.10 ± 0.36	13.23 ± 0.32	12.03 ± 0.35
120h	13.93 ± 0.31	13.43 ± 0.15	12.67 ± 0.15	13.97 ± 0.06	12.97 ± 0.25
240h	16.77 ± 0.21	16.17 ± 0.21	15.50 ± 0.21	16.37 ± 0.15	15.43 ± 0.21

In *Salmonella typhi*, the weakest antimicrobial activity was expressed after culturing *T. purpurogenus* in 24h that the inhibition zone was smallest and measured about 8.07 ± 0.15 mm (Table 1). The activity increased until culturing in 240h, showing the highest activity. The inhibition zone was measured about 16.77 ± 0.21 mm (Table 1).

In *Pseudomonas aeruginosa*, the weakest antimicrobial activity was expressed after culturing *T. purpurogenus* in 24h that the inhibition zone was smallest and measured about 7.23 ± 0.25 mm (Table 1). The activity increased until culturing in 240 h, showing the highest activity. The inhibition zone was measured about 16.17 ± 0.21 mm (Table 1).

The weakest antimicrobial activity against *Listeria monocytogenes* was expressed after culturing *T. purpurogenus* in 24h that the inhibition zone was smallest and measured about 8.43 ± 0.15 mm (Table 2). The activity increased until culturing in 240h, showing the highest activity. The inhibition zone was measured about 16.37 ± 0.15 mm (Table 1).

In *Serratia marcescens*, *T.purpurogenus* showed the weakest antimicrobial activity after 24h of culturing that the inhibition zone was smallest and measured about 6.87 ± 0.32 mm (Table 2).

The activity increased until culturing in 240 h, expressing the highest activity. The inhibition zone was largest and measured about 15.50 ± 0.21 mm (Table 1).

T. purpurogenus expressed the weakest antimicrobial activity against Vibrio *parahaemolyticus* after 24 h of culturing where the inhibition zone measured about 5.23 ± 0.25 mm (Table 2). After 240 h of culturing, the activity increased, showing the highest activity where the inhibition zone was measured about 15.47 ± 0.50 mm (Table 1).

From the results of antimicrobial activities determined in different incubation time that was summarized in Table 1, the analysis of the effects of incubation time on the antimicrobial activities of *T. purpurogenus* was done (Figure 1).

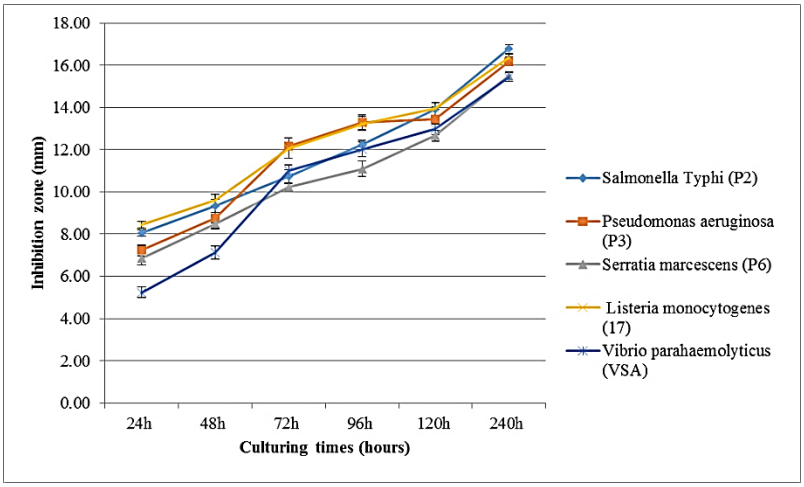

Figure 2: Antimicrobial activity of *T .purpurogenus* on different strains of pathogens

From Figure 2, the results exhibited that antimicrobial activity of the *T. purpurogenus* continuously increased from 24 hours to 240 hours of culturing. This species showed the best antimicrobial activity at 240 hours with all types of pathogens.

Preliminary Purification

Because *T. purpurogenus* extract showed biological activity, the study tried to perform the preliminary purification for understanding about characteristics of the biological agents. Petroleum ether and chloroform fractions were collected, evaporated and dispersed into distilled water. The powders obtained in petroleunm ether and chloroform fractions could be soluble in water. As a result, petroleum ether and chloroform fractions could contain certain compounds which were soluble in both polar and non-polar solvents.

Antimicrobial Test of the Purified Substance

In order to test which fraction contained the antimicrobial agent, the antimicrobial tests were done. The partial purified substances in chloroform fraction had the antimicrobial activity with *Salmonella typhi* (P2) and *Listeria monocytogenes* (17) (Figure 3). However, it showed no zone of inhibition with *Pseudomonas aeruginosa* (P3), *Serratia marcescens* (P6), *and Vibrio parahaemolyticus* (VSA).

Salmonella typhi and *Listeria monocytogenes* with different purification ratios using chloroform for extraction. From Figure 3, it was seen that the inhibition zones which exhibited the antimicrobial activity of the purified substances became larger when the ratio chloroform: supernatant increase from (1:2) to (2:1). In contrast, the inhibition zones of the supernatant after purification become smaller when the ratios chloroform: supernatant increase with the same ratio.

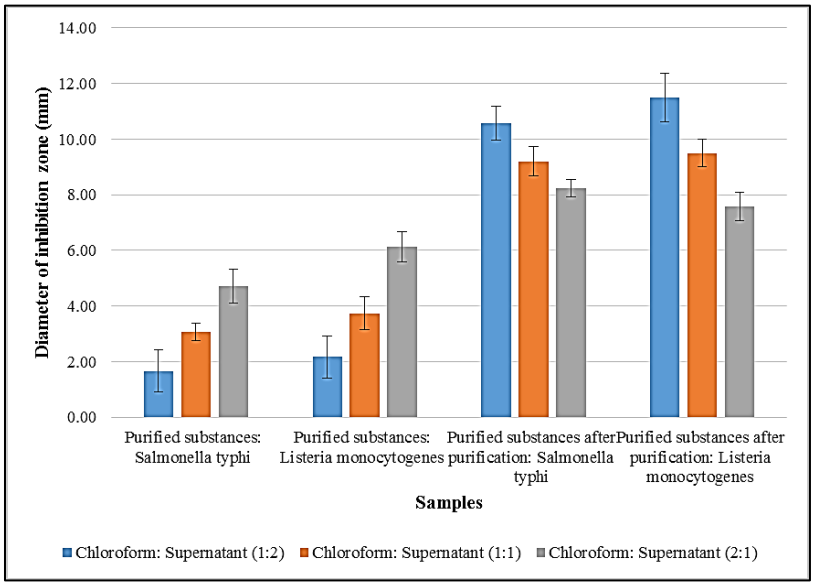

Figure 3: Antimicrobial activities of purified substance and the supernatant after purification against

Cytotoxicity activity on Hela Cells

The initial concentration of the stock solution was 1 g/mL. It was diluted to the concentration of 100mg/mL in order to perform the test. The test showed no cytotoxic activity on Hela, in contrast, showed high cytotoxic percentage at the concentration of 100 mg/mL (97.29 ± 0.99%). The extract was diluted into different concentrations to determine the half maximal inhibitory concentration (IC50) on Hela cancer cell line. Result of this test was recored in Table 2.

Table 2: Cytotoxic activity and the half maximal inhibitory concentration (IC50) of *T. purpurogenus* on Hela cancer cell lines

Concentration (mg/mL)	Cytotoxic percentage (%)			
	1st time	2nd time	3rd time	Mean ± SD
100	96.15	97.8	97.92	97.29 ± 0.99
75	94	94.4	93.75	94.05 ± 0.33
50	58.89	62.8	65.28	62.32 ± 3.22
25	17.49	17.72	16.5	17.24 ± 0.65
12.5	3.54	5.97	1.16	3.55 ± 2.41
IC50 (mg/mL)	49.02	47.5	48.36	48.29 ± 0.76

T. purpurogenus showed the weakest cytotoxicity to MCF-7 cancer cell line (0.45 ± 4.30%) while the strongest cytotoxic percentage was expressed at Hela (97.29 ± 0.99%). IC50 of *T. purpurogenus* on Hela cancer cell lines was 48.29 ± 0.76 (mg/mL).

DISCUSSION

YM medium was chosen for culturing *T. purpurogenus* because it is rich in nutrients. YM provided fungus with two important nutrient sources, carbon source (in dextrose, beef extract) and protein source (in beef extract and yeast). In addition, the pH of medium is 6.5, nearly equal to the pH required for culture fungi medium. From Figure 1 and

Table 1, it could be concluded that *T. purpurogenus* was able to produce substances with antimicrobial activity when cultured in YM medium.

As showing in Figure 1, more than one antimicrobial agents in *T. purpurogenus* supernatant because the activities did not form a straight curve when culturing from 24 h to 240 h. In the case of *Pseudomonas*, the activity increased from 24 h to 72h, then the activity looked stable until 120 h, but sudden increased until 240 h. It was meant that there was a complexity of antimicrobial activities in *T. purpurogenus*. To identify the antimicrobial compounds, more studies should be done so far. During preliminary purification, after using petroleum ether extraction, the antimicrobial activities was not detectable. The supernatant layer stayed below the petroleum ether layer and above the chloroform layer. From Figure 3, the results indicated that there are more than one substance with having antimicrobial activity are produced during the time of culturing *T. purpurogenus*.

CONCLUSION

In fact, the supernatant remained the antimicrobial activities against all pathogens even though the chloroform gave the antimicrobial activities against *Salmonlla typhi* and *Listeria monocytogenes*. The more amount of chloroform, the more substances were purified. The inhibition zones were also greater. The inhibition zone of the supernatant became smaller each time the amount of chloroform solvent increase. It means the supernatant has lost substance during the purification using chloroform and that substance has laid in the chloroform layer. With the concentration 100 mg/mL, the supernatant exhibited high cytotoxic ability with Hela cancer cell line (97.29 ± 0.99%). IC50 was detected at 48.29 ± 0.76 (mg/mL). The mechanism and new agent will identified so far.

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