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Cytotoxic activity screening of ethyl acetate fungal extracts derived from the marine sponge *Neopetrosia chaliniformis*AR-01

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ABSTRACT

This research explored the potential of fungi derived from the marine sponge *Neopetrosia chaliniformis* as producers of cytotoxic compounds. Fungi derived from *N. chaliniformis* were isolated by cultivating on Saboroud Dextrose Agar (SDA) and purified by streak plating. The pure isolated fungi were cultivated using rice as media at 25-27 °C for 4-8 weeks and extracted using ethyl acetate solvent. The ethyl acetate extracts were tested for cytotoxicity using an MTT assay against colon cancer cells (WiDr) and normal cells (VERO). The fungal isolates which have the greatest cytotoxic activity were identified based on morphological and molecular characterization. Thirteen isolates of the fungus were obtained from *N. chaliniformis*. Six fungi isolates labeled NC01, NC02, NC03, NC06, NC07, and NC09 were found to be cytotoxically active with percentage of viability against WiDr colon cancer cells of 12.1, -2.43, 29.02, 70.31, 14.04, and 46.04, respectively. However, only NC06 isolate that was identified as *Aspergillus nomius* with a homology percentage of 99 % based on molecular characterization using 18S rRNA primer showed non-cytotoxic activity against normal Vero cells with percentage of viability of 113.99%.

INTRODUCTION

Marine sponges are hosts to various bacteria and fungi. (Hentchel *et al*., 2006; Wang, 2006). But the type of relationship and ecological function of these microorganisms in the sponges remain unclear (Wiese *et al*., 2011). However, some, especially fungi, are known to produce potentially bioactive compounds that could be used to combat cancer and pathogenic microbial infections (Wiese *et al*., 2011; Bhadury *et al*., 2006; Vasanthabharathi and Jayalakshmi, 2011). The marine sponge *Neopetrosia chaliniformis* is an endemic sponge found along the Indonesian coastline whose medicinal potential is only beginning to be investigated. In our previous research, we isolated 13 fungi derived from *N. chaliniformis* and over three-quarters of these produced cytotoxic isolates with LC_{50}<100 ppm using a Brine Shrimp Lethality Test. We also reported on fungi isolates that showed antibacterial activity against *B. subtilis*, *S. epidermidis*, *S. typhosa* and *E. coli* (Handayani and Artasasta, 2017). This present research is a continuation of the effort to identify bioactive compounds from marine sponge-derived fungi.

MATERIALS AND METHODS

Sponge material, isolation, cultivation and extraction of Secondary Metabolites from Fungi Isolates associated with marine sponges

The sponge material which was used and the stages of the research method for isolation and cultivation of fungi were carried out as in our previous study (Handayani dan Artasasta, 2017).

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MTT Assay

Sample Screening

Two cell lines, WiDr as a colon adenocarcinoma cell line, and Vero as normal cell line were prepared for cytotoxic assay using MTT. All cell lines were obtained from Laboratory of Parasitology at Gajah Mada University, Yogyakarta, Indonesia. WiDR was cultured in RPMI 1650 and Vero was cultured in M199 Medium. All cells were subcultured after mild trypsinization with trypsin-EDTA (Sigma-Aldrich, USA), and then the cell number and viability were determined. The cells were seeded onto 96-well plates at a density of $6x10^3$ cells/well in 100 µL medium and incubated overnight. All media were supplemented with 10% fetal bovine serum (Gibco) and streptomycin and penicillin (2%, Sigma-Aldrich, USA). The cell line were kept at 37ºC, 98% relative humidity with 5% CO_2 atmosphere.

A stock solution was prepared by dissolving the samples in DMSO at 100,000 ppm. Cells that had been incubated 24 hours were divided into groups; treatment, positive control, cell control and media control (blank). The medium was removed and the cells washed using 100 µL sterile PBS in each well. Then, 100 µL of each material (extract) at concentration of 100 ppm was added to each well. Doxorubicin was used as the positive control. Each extract was incubated for 24 hours at 37 ° C, 5% CO_2 then mixed throughout the medium and washed using sterile PBS. Then 100 mL of MTT (5mg / ml) was added to each well followed by a 4-hour incubation at 37 ° C, 5% CO_2. 100 µL of 10% SDS was added to each well to dissolve the formazan crystals formed followed by overnight incubation at room temperature. The plates were then read by an ELISA reader at 540 nm (Permanasari *et al*., 2016). The aim of this screening was to identify the presence of cytotoxic activity.

Cytotoxic Screening

The cytotoxicity on WiDr cells of the EtOAc fungal extracts derived was determined by the MTT assay. 5 x 10^3/well cells were plated with 100 µL of medium/well in 96-well plates. After overnight incubation in a humidified atmosphere with 5 % CO_2 at 37 ^oC, the fungal extract was added in concentrations of 100 ppm. 100 µL of 0.5 mg/mL MTT (pH 4.7) was then added to each well and cultivated for another 4 hours. The reaction was stopped by 100 µL SDS (10 %) in HCl 0.01 N per well. After overnight incubation, the absorbance at 595 nm was measured with an ELISA reader (Bio-Rad), using wells without cells as blanks. All experiments were performed in triplicate. Doxorubicin was used as a positive control. The effect of each fungal extract on the proliferation of colon cancer cells was expressed as the percent viability.

Molecular Identification

DNA Extraction

DNA extraction was conducted by following the method of Saitoh *et al.* (2006). Mycelia of fungal isolate were excised with a toothpick from a 4-6 day-old culture plate and transferred to a microtube with 500 µL of lysis buffer. The mycelia were dispersed in the buffer with a toothpick and incubated for 10 min at room temperature. The mixture was centrifuged at 18000 rpm for 5 min at 4^{o}C. The supernatant was transferred to a fresh micro-tube and mixed with 750 µL of ethanol by inverting the tube, the DNA was precipitated by centrifugation at 18000 rpm for 2 min at 4^{o}C. The DNA pellet was dried than dissolved in 50 µL of TE buffer pH 8.0.

PCR Amplification and Sequencing of 18S rRNA-encoding gene

The PCR mixture (50 µL) containing 1 µL of the DNA template, 20 µL dH_2O, 25 µL PCR master mix, 2 µL primer 18F (5' -ATC TGG TTG ATC CTG CCA GT-3') and 2 µL primer 18R (5' -GAT CCT GCA GGT TCA CC-3'). Amplification reactions were performed in a Hybaid Omnigene thermal cycler. An initial denaturation for 2 min at 94 ^{o}C was followed by 30 cycles of 15 sec at 94 ^{o}C, 30 sec at 60 ^{o}C and 1.30 min at 68 ^{o}C and final extension of 10 min at 78 ^{o}C.

PCR products were sequenced in First Base, Malaysia. The sequences were analyzed by the BLASTN program on NCBI. Analysis of phylogenetic trees was constructed using neighbor-joining tree method with a bootstrap value of 1000 replication by using MEGA 7.0 software (Tamura *et al*., 2016).

RESULTS AND DISCUSSION

Screening of cytotoxic activity used an MTT assay to measure cytotoxicity to WiDr colon cancer cells compared to VERO normal cells. The optimally performing extract was classified by its toxicity to cancer cells while being non-toxic to normal cells. The 100 ppm EtOAc extract of the NC06 isolate was the most selectively cytotoxic extract on WiDr colon cancer cells and VERO normal cells with percentage of viability being 70.31 % and 113.99 %, respectively (Table 1). The NC06 isolate was then identified using molecular characterization by partial sequence analysis of the 18S rRNA gene.

Table 1: The cytotoxic activity of EtOAc fungal extract 100 ppm derived from marine sponge *N. chaliniformis* by using MTT method.

No	Fungal Isolates Code	Percentage of viability (%)	
		WiDr	VERO
1	NC01	12.11	-5.57
2	NC02	-2.43	26.56
3	NC03	29.02	-1.73
4	NC04	-	-
5	NC05	110.44	-1.58
6	NC06	70.31	113.99
7	NC07	14.04	21.14
8	NC08	95.52	128.14
9	NC09	46.04	19.48
10	NC10	106.85	137.77
11	NC11	109.61	136.94
12	NC12	-	-
13	NC13	-	-
Positive Control	Doxorubicin	16.42	96.68

The 18S rRNA-encoding gene was amplified using PCR using universal primers 18F (5 '-ATC TGG TTG CTC CCA GT-3') and 18R (5 '-GAT CCT GCA GCA TCA CC-3'). DNA bands of 1690 bp were obtained (Figure 1). A BLAST search in NCBI-

GenBank showed that the NC06 isolate had a maximum identity of 99 % with *Aspergiilus nomius.*

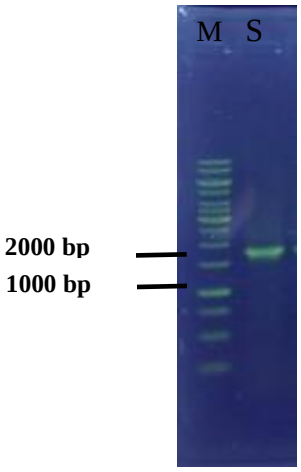

Fig. 1: PCR product of 18S rRNA gene from NC06 isolate derived from *N. chaliniformis.* There is a 1690 bp band from the sample and the marker is 1 kb.

The phylogenetic tree was constructed using a neighbor-joining method with a bootstrap value of 1000. The result of analysis showed that tree formation consists of two clades. Clade 1 was a group of *Aspergillus* and clade 2 was a group of *Penicillium.* NC06 isolate was on clade 1 confirming its identity as *Aspergillus nomius* (Figure 2).

Cytotoxic screening is conducted using a cancer cell line to determine the specific sensitivity of the effect of the bioactive compound. In this study, cytotoxic screening was performed using a WiDr colon cancer cell line and the cytotoxic effect compared with normal VERO cells. The WiDr colon cancer cell line was chosen because it can be cultured easily and repeatedly for cytotoxic testing (Rohmah *et al*., 2013).

In this research, we used an MTT assay. This is a colorimetric method, in which the MTT reagent is a tetrazolium salt that can be broken down by succinate-tetrazolium reductase, a mitochondrial enzyme, to produce purple formazan crystals. The absorbance of these crystals can then be measured using an ELISA reader (Doyle and Griffith, 2000; Berrigde *et al*., 1993).

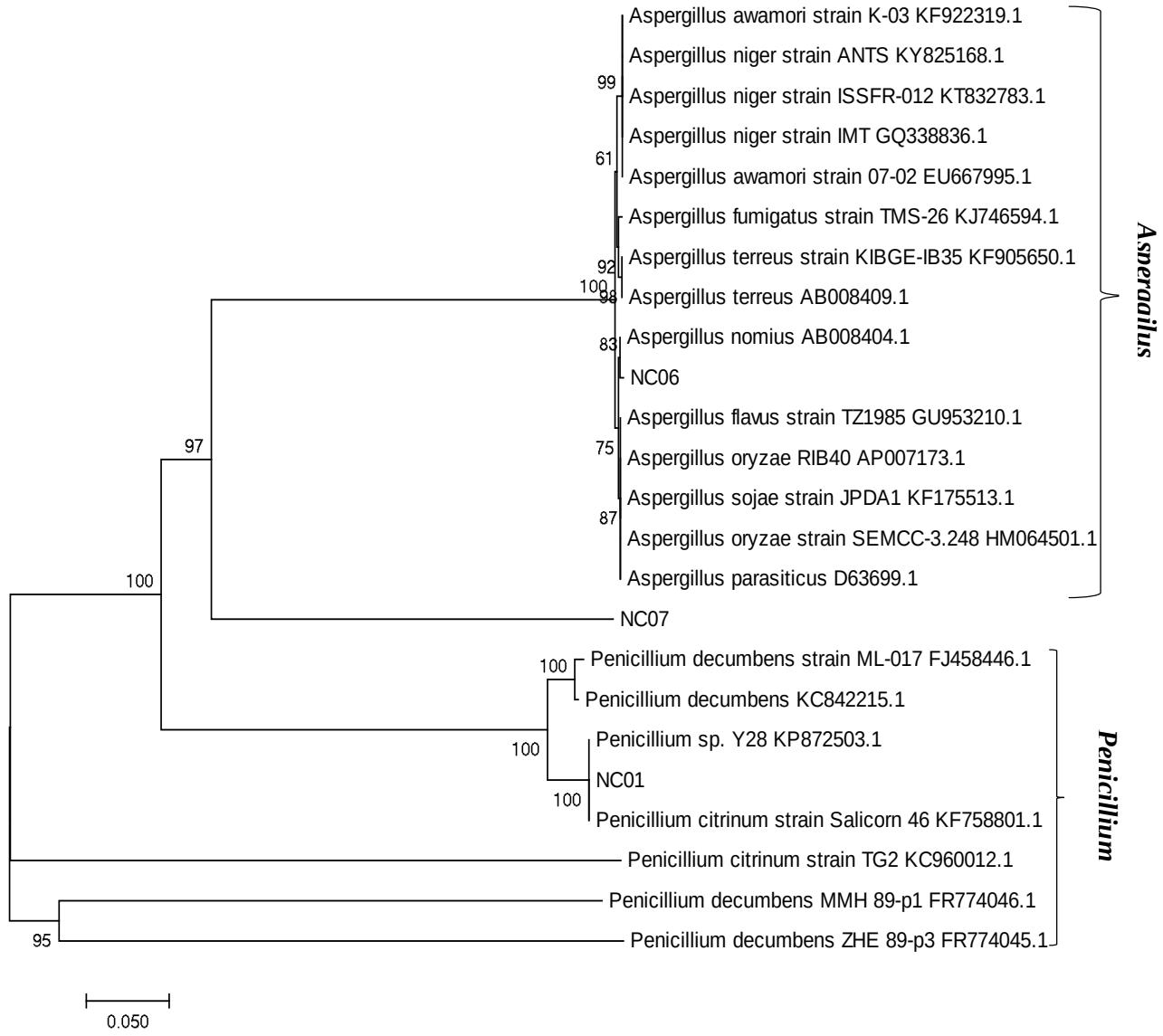

Fig. 2: The phylogenetic tree by using neighbour-joining method of 18S rRNA gene sequence from some of fungi isolate derived *N.chaliniformis* and some strain of *Aspergillus* and *Penicillium.* The scale bar indicates a 0.05 substitution nucleotide position.

The optimally performing extract was classified by its toxicity to cancer cells while being non-toxic to normal cells. The 100 ppm EtOAc extract of the NC06 isolate was the most selectively cytotoxic extract on WiDr colon cancer cells and VERO normal cells with percentage of viability being 70.31 % and 113.99 %, respectively. Gomes, et el., (2015) revealed that a cytotoxic compound can be a potential anticancer agent when it shows selectivity between normal and cancer cell lines, exhibit activity against multidrug-resistance (MDR), and demonstrates a cell death mechanism through inhibiting non-apoptosis.

Based on the reconstruction of the phylogeny tree in Figure 2. NC06 isolate shows 99% homology with *Aspergillus nomus.* Little is known about the effects of bioactive compounds from *A. nomius* on WiDr colon cancer cells. However potential anti-cancer activity against human colon cancer cell lines has been demonstrated. For example, Lee *et al*., (2010) showed some compounds from *Aspergillus versicolor* derived from *Petrosia sp* had IC_{50} against HCT15 colon cancer cell line below 30 µg/mL. Chol *et al*., (2010) found that diketopiperazine disulfides produced by the marine fungus *Aspergillus sp* KMD 901 had potential to induce apoptosis on HCT116 colon cancer line. Avrainvillamide derived from marine *Aspergillus sp.* CNS358 has a dose-dependent cytotoxic effect against human colon cancer cell HCT116 with IC_{50} of 2.0 µg/mL (Fenical *et al*., 2000). *Aspergillus sp.* derived from marine sediment was found to contain the peptide aspergillamide, that had a mild cytotoxic effect on human colon carcinoma HCT-116 cell with IC_{50} of 16 µg/mL (Toske *et al*., 1998).

The secondary metabolites that have been isolated from *Aspergillus nomius* are nominine, aspernomine, 14-hydroxipaspalinine, and 14-(N, N-dimethyl-L-valyloxy) paspalinine. These compounds are Indole alkaloids derived from cyclic dipeptides which have bioactivity as insecticides (James and Rinderknecht, 1989; Staub *et al*., 1992; Staub *et al*., 1993). However, one strain of fungus can produce diverse secondary metabolites depending on growth media and source. (Bode *et al*., 2002). Therefore, the NC06 isolate from *Neopetrosia chaliniformis* that showed similarity to *Aspergillus nomius* may produce other secondary metabolites just as the fungus *Dothideomyceta sp.* CR17 produced diverse compounds in different media (Senadeera *et al*., 2012). When cultivated in PDB medium prepared from fresh potato tubers, CR17 produced azaphilone derivatives such as dothideomycetones A, B and dothideomycetide A with a tricyclic polyketide but upon cultivation in PDB medium prepared from a commercial potato powder it produced calbistrin F, G and H.

CONCLUSION

Marine derived fungus NC06 from sponge *N. chaliniformis* AR-01 showed the most selective cytotoxicity against WiDr cell line compared to the Vero cell line. Molecular characterization indicated that this NC06 fungus was *Aspergillus nomius.* Further research needs to be conducted to identify the cytotoxic compounds produced by the fungus.

CONFLICT OF INTERESTS

Declared none

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Conflict of Interests: There are no conflicts of interest.

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