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Screening of cytotoxic activities toward WiDr and Vero cell lines of ethyl acetate extracts of fungi-derived from the marine sponge *Acanthostrongylophora ingens*

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ABSTRACT

Ethyl acetate extracts of fungi-derived from the marine sponge *Acanthostrongylophora ingens* were tested for cytotoxic activity against WiDr and Vero cell lines. Three of fungi extracts exhibited strong cytotoxicity with percentage of viability (≤50%) occurring at concentrations of 100 µg/ml. One isolate (IB141) showed specific cytotoxicity against WiDr cells whreas not against Vero cells. This isolate was identified based on molecular characterization using sequence analysis of the partial 18S rRNA gene. The result indicated that IB141 was identical to *Aspergillus ochraceus*. A comparatively high part of positive bio-activity screening results were acquired in this study, displaying that the fungi-derived from the marine sponge *A. ingens* have potential as a source of new anti-cancer agents.

INTRODUCTION

Marine-derived microbial communities have been focused more in the recent research. The species composition is diverse and shows temporal and geographical variations (Webster *et al*., 2001; Brigitte, 1980). The bacteria and fungi in these communities are the potential source of a wide range of bioactive natural products (Rateb and Ebel, 2011; Subramani *et al*., 2013; Thomas *et al*., 2010). Marine sponges have repeatedly been shown to contain previously unknown bioactive strains of fungal species (Pitt, 2000; Sun *et al*., 2012; Wiese *et al*., 2011). It should be noted that the same fungal species from a different sponge species produced dissimilar secondary metabolites (Jadulco *et al*., 2002). These sponges are the single most productive source of marine fungi that producing large numbers of bioactive compounds and secondary metabolites found to date (Bugni and Ireland, 2004; Bhadury *et al*., 2006; Proksch *et al*., 2003). In addition, many of these secondary metabolites are novel compounds with anti-cancer, anti-inflammatory, anti-microbial, or anti-viral properties (Elsebai *et al*., 2011; Lee *et al*., 2013; Li *et al*., 2013).

Cancer is a frightening human disease, increased along by changes in lifestyle, nutrition, and environmental conditions (Jemal *et al*., 2011; Marmot *et al*., 2007; Veer and Kampman, 2007). The treatment of cancer does not hold an effective medicine because the currently available drugs are producing severe side effects. The advances in the knowledge of cancer biology have made it possible to expand strategies in finding new anti-cancer drugs. It is of interest to record that further than 50% of the drugs applied in the therapy of cancer come from nature (Boopathy and Kathiresan, 2010). The natural products derived from the ocean are a significant source to discover new anti-cancer agents, including marine-derived fungi. Fungi derived from marine to prove become a kind candidate as a resource of new anti-cancer

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compounds. For continuing our work on bioactive secondary metabolites produced by fungi-derived from mangrove plants and marine sponges (Handayani and Artasasta, 2017; Handayani *et al*., 2016; 2017; 2018), a preliminary study on the anti-bacterial and cytotoxic activities of ethyl acetate extracts of fungi-derived from the marine sponge *Acanthostrongylophora ingens* has been conducted (Handayani and Aminah, 2017). This provided study is a continuity of the attempt to find out the cytotoxic activity of ethyl acetate fungi extracts against a WiDr cancer cell line and Vero normal cells.

MATERIALS AND METHODS

Sponge material

Acanthostrongylphora ingens is taken from the sea around Mandeh Island, West Sumatra, from a depth of ±5–8 m. It was transferred to a sterilized plastic bag and borned in an ice box for transfer to the laboratory. Taxonomic identification of this sponge has been identified by Dr. Nicole J. De Voogd, at the Natural Biodiversity Center, Netherlands. A voucher specimen, named IB101, has been conserved in the Marine Reference Collection, the Biota Sumatra Laboratory, Andalas University, West Sumatra, Indonesia.

Isolation and cultivation of fungi-derived from sponge

The sterilized sponge surface by rinsing with sterile seawater was cut into small pieces. Ten grams were entered into an Erlenmeyer flask with 100 ml of sterilized seawater then more seawater was added to produce a 10–6 dilution. The resulting mixture was used to inoculate sabouraud dextrose agar and incubated at 27°*C*–29°*C for 5*–7 days. Colonies were differentiated by shape and color and purified by pouring method to get pure isolates. The pure isolates were carried out based on Brigitte methods (1980). The pure isolates of fungi derived from the sponge were cultivated in a rice medium and then incubated for 4–6 weeks at room temperature until the medium was covered with the fungi (Kjer *et al*., 2010).

Extraction of secondary metabolites from fungi isolates

Ethyl acetate was then extracted from each of these fungal isolates with ethyl acetate (EtOAc). The EtOAc extracts were collected and evaporated *in vacuo* using a rotary evaporator then tested for cytotoxic activity using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay against WiDr and Vero cell lines.

Cytotoxic screening

Cell line

WiDr cancer cells and Vero normal cells were obtained from CCRC, Faculty of Pharmacy, Gadjah Mada University. These cells were preserved in RPMI-1640 amplifier with 10% FBS (Fetal bovine serum) , penicillin (10,000 U/ml), streptomycin (10 mg/ml), and fungizone (0.5 ml) in a moistened atmosphere of 50 µg/ml CO_2 at 37°C.

MTT assay

The cytotoxicity of the ethyl acetate extracts of fungi against WiDr cancer cells was tested by MTT assay. In 96-well plates, 5×10^3/well of cells were gilded in 100 µl of medium/well. Then it was incubated overnight in a moistened atmosphere with 5% CO_2 at 37°C and the extracts with concentration of 100 ppm were added. After addition of the extracts, 100 µl of 0.5 mg/ml MTT (pH 4.7) per well and the plates cultured for 4 hours. The reaction ended with 100 µl SDS 10% in 0.01 N HCl per well and incubated overnight. The absorbance at 595 nm was measured using an ELISA (Enzyme-Linked Immunosorbent Assay) reader (Bio-Rad). Doxorubicin was formed as a positive control. All trials were done in triplicate (Permanasari *et al*., 2016). The results of fungal extract on the growth of WiDr cancer cells were avowed as a percentage of viability was given as follows:

$$\text{\% Cell viability then calculated by the equation} = \frac{\text{OD of treatmnet} - \text{OD of blank}}{\text{OD of control} - \text{OD of blank}} \times 100\%$$

Molecular identification

DNA extraction

The pure fungal isolates were cultured 4–6 days, the micelles were cut using toothpick and transferred to a microtube containing 500 µl of lysis buffer. Then incubated at the room temperature of 10 minutes. The blend was centrifuged for 5 minutes at 4°C with 18,000 rpm. The resulting supernatant was transferred to a sterile microtube and added with 750 µl of ethanol then homogenized by turning the tube. The DNA was centrifuged for 2 minute at 4°C with 18,000 rpm. The results of dried DNA pellets after cleaning with 70% ethanol were dissolved in 50 µl TE buffer pH 8.0 (Saitoh *et al*., 2006).

PCR amplification and sequence of 18S rRNA gene

The 50 µl PCR blend contained 1 µl DNA template, 20 µl dH_2O, 25 µl PCR control 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′). The amplification responses were ruined in a Hybaid Omnigene thermal cycler. An initial denaturation was carried out for 2 minutes at 94°C and followed by 30 cycles for 15 seconds at 94°C, 30 seconds at 60°C and 1.30 minutes at 68°C and a last prolongation of 10 minutes at 78°C. The sequences were examined by the BLAST program on NCBI. Phylogenetic tree analysis was assembled by the neighbor-joining method to a bootstrap value of 1,000 replications using MEGA 7.0 software (Kumar *et al*., 2016).

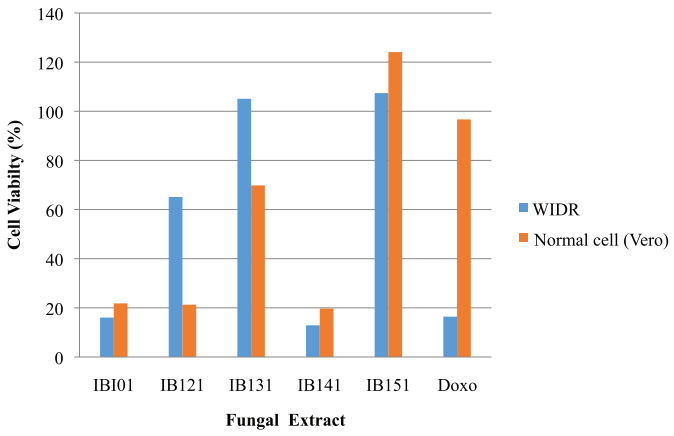

Figure 1. Cytotoxic effect of extract fungi from sponge A. ingens on WiDr and normal cell (Vero) lines.

RESULTS AND DISCUSSION

The fungi extracts that showed potential cytotoxity with viability percentage (≤50%) against the WiDr and Vero cell lines were 1B101, 1B121, and IB141 (Fig. 1). IB141 was the highest cytotoxicity against WiDr cancer compared with Vero normal cells with the percentage of viability 12.88% and 19.71%, respectively. This IB141 isolate was identified at a molecular level by sequence analysis of the partial 18S rRNA gene.

The amplified PCR of the 18S rRNA gene showed that DNA bands were obtained at 978 bp. A BLAST search on the NCBI gene deposit showed that this IB141 isolate had a maximum identity of 99% with *Aspergillus ochraceus* strain UPSC 1983 (Table 1).

The phylogenetic tree assembled with a neighbor-joining method using a scale bar of 0.5 substitutions per nucleotide. The resulting tree consists of two clades (Fig. 2). *Eurotiales* (genera *Aspergillus*) are the dominant group with 24 representatives of the fungus. BLAST and phylogenetic analysis formed on the 18S rRNA gene sequences indicate that the IB141 isolate had a 90% identity with *Aspergillus unguis*. This is in agreement with the results of the BLAST search.

The IB141 isolate, which can be assumed to be *A. ochraceus* Strain UPSC 1983, was most selective cytotoxic to WiDr cancer and normal Vero cells found in this study.

Based on the results of phytochemical constituents showed that ethyl acetate extract from IB141 isolate contains phenolic and terpenoid compounds (Handayani and Aminah, 2017). In previous studies, alkaloids Stephacidin A and B isolated from *A. ochraceus* WC76466 were found to possess anti-tumor activity (Qian-Cutrone *et al*., 2002). The fungus *A. ochraceus* JGI25 was also the source of an alkaloid that had notable cytotoxicity against HeLa cancer cell with the IC_{50} value being a smaller extent than 40 µg/ml (Nadumane *et al*., 2013).

Table 1. Molecular identification of IB141 fungi derived isolate from sponge *A. ingens* based on 18S rRNA gene.

Isolate code	Species	Homology (%)	Query length	Access number
IB141	*Aspergillus ochraceus* Strain UPSC 1983	99	978	LPB_141

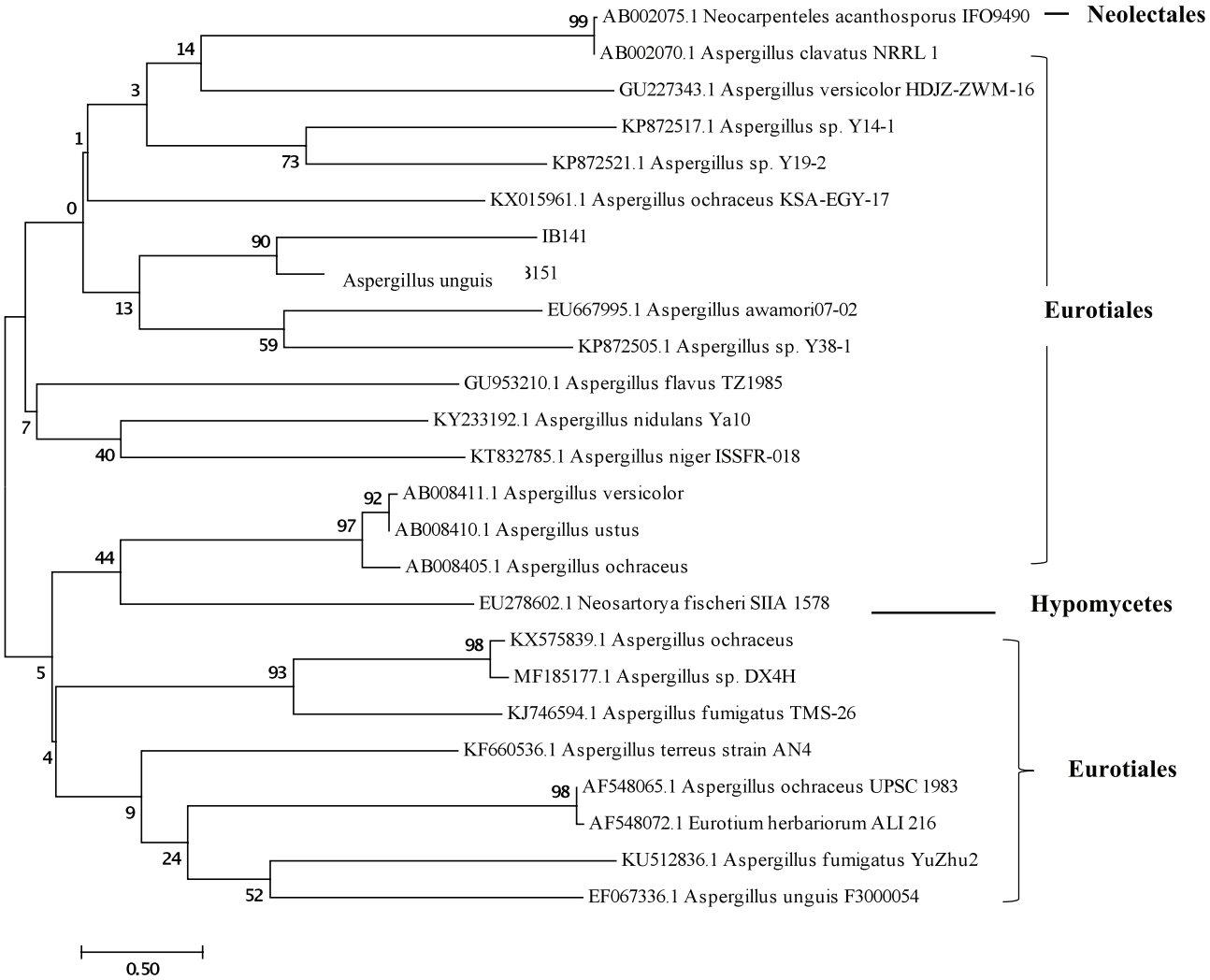

Figure 2. Neighbor-joining phylogenetic tree of marine-derived fungi from sponge *A. ingens* and some strains of *Aspergillus* based on 18S rRNA gene sequences. The values at each node represent the bootstrap values from 1,000 replicates, and the *scale bar* represents 0.5 substitutions per nucleotide.

Identification of novel and new anti-cancer metabolites by using standard screening and isolation methods has proven that fungi from the genus *Aspergillus* have capability to bring out interesting bioactive compounds (Nadumane *et al*., 2013). For example, *Aspergillus versicolor* derived from *Petrosia* sp. showed some compounds derived from this fungus had IC_{50} for colon cancer cell of HCT15 lower than 30 µg/ml (Lee *et al*., 2010). Marine fungus *Aspergillus* sp. KMD901 contained diketopiperazine disulfide which can induce apoptosis in an HCT116 cancer cells (Chol *et al*., 2010). Phenylahistin, a diketopiperazine alkaloid was obtained from *Aspergillus ustus* (Kanoh *et al*., 1997) and exhibited cytotoxic activity against *in vitro* tumor A-549, A-431, K-562, HeLa, WiDr, MCF-7, and TE-671 cell lines, with IC_{50} values ranging from 0.18 to 3.7 µM (Kanoh *et al*., 1999).

In our previous research of anti-cancer–producing compound from marine-derived fungi has been performed, *Aspergillus nomius* derived of sponge *Neopetrosia chaliniformis* AR-01 indicated the highest cytotoxic activity against WiDr cell line with percentage of viability of 70.31% (Artasasta,*et al*., 2017), and 16 extracts of derived fungi from marine sponge *Haliclona fascigera* (80%) obtained cytotoxicity against HeLa, WiDr, and T47D (Handayani *et al*., 2017). However, although marine-derived fungi are still had to understudy groups of marine ecological and cognition of regarding the diversity and potential anti-cancer function of fungi derived from the marine sponge *A. ingens* is still very limited. A comparatively high part of positive bio-activity screening results were acquired in this study, applying that fungi derived of the marine sponge *A. ingens* have potential as sources of new anti-cancer compounds.

CONCLUSIONS

In this study, fungal extract of IB141 derived from marine sponge *A. ingens* was the highest cytotoxicity against WiDr and Vero cell lines. Based on molecular identification result indicated that IB141 was identical to *A. ochraceus*. The cytotoxicity of this fungus has the potential as an alternative source for new anti-cancer compounds.

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