

# Antimicrobial and cytotoxic activities of marine sponge-derived fungal extracts isolated from *Dactylosporgia* sp.

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## ABSTRACT

This study is the first report on the antimicrobial and cytotoxic activities of fungal extracts isolated from marine sponge *Dactylosporgia* sp., which is collected from Mandeh Island, West Sumatra, Indonesia. The isolation of fungal was conducted using dilution method with Sabouraud Dextrose Agar + chloramphenicol (0.05%) as a medium. The pure isolated fungal was cultivated on rice medium at temperature 25°C–27°C and then extracted using ethyl acetate solvent. The ethyl acetate extract of each isolated fungal was tested for antimicrobial and cytotoxic activities. Nine fungal strains have been isolated from this sponge. Two ethyl acetate extracts of fungal strains (Dc03 and Dc04) were categorized as having strong inhibition against the growth of *Staphylococcus aureus*, *Escherichia coli*, methicillin-resistant *S. aureus*, and multidrug-resistant *Pseudomonas aeruginosa* in a concentration of 5% with zone inhibition in range of 12.31 ± 0.54–16.14 ± 0.75 mm. The cytotoxic activity screening of the ethyl acetate extracts of fungal strains was done by using the brine shrimp lethality test. Four fungal strains had LC<sub>50</sub> below 80 µg/ml (Dc03, Dc04, Dc05, and Dc08) and were further tested with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay on T47D cell line. These selected fungi were identified molecularly as *Cladosporium halotolerans* MN859971, *Penicillium citrinum* MN859968, *Aspergillus versicolor* MN859970, and *Aspergillus sydowii* MN859969, respectively. The results suggest that these fungal strains are quite rich in the production of bioactive compounds that are very effective as antibacterial and cytotoxic agents.

## INTRODUCTION

Marine-derived fungi have attracted much attention in recent years due to the production of structurally unique pharmacological properties (Blunt *et al.*, 2007; 2015). Fungi have proven as promising sources of new compounds compared to other microbial sources such as bacterial isolates from the sea (Gamal-Eldeen *et al.*, 2009). The first report of bioactive compounds from marine-derived fungi was cephalosporin C,

which was isolated from *Acremonium chrysogenum* that associated with a sewage outlet in the Mediterranean Sea close to the Sardinia Island. Next, cephalosporin C was developed as a lead compound of modern cephalosporin antibiotics for the treatment of numerous infections (Abraham and Loder, 1972).

Many researchers, nowadays, have developed the study which is focused on the biological activity of marine-derived compounds. Screening for antimicrobial and cytotoxic activity of marine-derived compounds is becoming more popular due to the search for novel antibiotic and antitumor agents. Hu *et al.* (2015) reported, in their study, that of 4,196 biologically active compounds isolated fungi from a marine organism, 2,225 had anticancer activities (56%) and 521 had antibacterial activities (13%).

Some symbiotic fungi of marine sponge in West Sumatra, Indonesia, had been reported to produce bioactive

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compounds with various biological activities. Fungi associated with marine sponge *Haliclona fascigera* were reported active toward pathogenic bacteria such as *Staphylococcus aureus* and *Candida albicans* (Handayani *et al.*, 2015a; 2015b). Marine sponge *Acanthoryngylophora ingens* and *Neopetrosia chaliniformis* AR-01-derived fungi collected from Mandeh Island, West Sumatra, Indonesia, showed an inhibitory activity against *Bacillus subtilis*, *Staphylococcus epidermidis*, *Salmonella typhi*, and *Escherichia coli*. The screening for cytotoxic activity using brine shrimp lethality test (BSLT) method against brine shrimp larvae also showed a significant LC<sub>50</sub> value (Handayani and Aminah, 2017a; Handayani and Artasasta, 2017b).

In the study of marine-derived microbial in West Sumatra, Indonesia, we had reported the potential marine sponge-derived bacteria (Handayani *et al.*, 2015a; 2015b; 2016; Sandrawati *et al.*, 2019), marine sponge-derived fungi (Handayani *et al.*, 2018a; 2018b; 2019b), and algae-derived fungi (Handayani *et al.*, 2019a) which displayed interesting biological activities. In this present study, we described the isolation of fungi associated with marine sponge *Dactylosporgia* sp. which was cultivated on a rice medium. We also evaluate the antimicrobial and cytotoxic activities of its EtOAc extracts and the identification of fungi by microscopic and molecular determination.

## MATERIALS AND METHODS

### Sample material

Sponge sample code DH3 was collected from the Mandeh Island, West Sumatra, in a depth of ±10 m. It was surface sterilized by sterile seawater three times and stored in a plastic bag. The sample was placed in an icebox and transferred to the laboratory immediately for the isolation of symbiotic fungi.

### Identification of sponge

Sponge identification was conducted by Dr. Nicole J. De Voogd at the Natural Biodiversity Center, the Netherland. The sponge code DH3 was identified as *Dactylosporgia* sp.

### Isolation, cultivation, and extraction of symbiotic fungi

The method of isolation and cultivation of symbiotic fungi was carried out in the previous research (Kjer *et al.*, 2010). The symbiotic fungi were macerated with ethyl acetate in the ratio 1:1 with three repetitions. The resulting extract was then evaporated *in vacuo* with a rotary evaporator and used for the antimicrobial and cytotoxic assays.

### Screening for antimicrobial activity

The EtOAc extract of fungus was tested using pathogenic microbial as follows: *S. aureus* ATCC25923, *E. coli* ATCC25922, *C. albicans*, and clinical isolate methicillin-resistant *Staphylococcus aureus* (MRSA) which were obtained from Cipto Mangunkusumo Hospital, Jakarta, Indonesia, and multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) was from M. Djamil Hospital, West Sumatera, Indonesia.

### DISK DIFFUSION METHOD

The EtOAc extract of fungus was prepared for the concentration of 1, 3, and 5 mg/ml in dimethyl sulfoxide (DMSO) solvent. About 10 µl of each EtOAc extract was pipetted onto a

6-mm sterile paper disk (Advantec®). Each of the disk was put on the surface of the Sabouraud Dextrose Agar (Merck®) medium containing 0.7 McFarland of fungi suspension and on the surface of Natrium Agar (Merck®) contained 0.5 McFarland of bacterial suspension. Chloramphenicol (Oxoid®) in a concentration of 30 µg/ml was used as a positive control for antibacteria and nystatin (Oxoid®) in a concentration of 100 unit/disk as antifungi. The paper disk was also inoculated with 100 µl DMSO as a negative control. Each treatment consisted of three replicates.

The plates were incubated overnight at 37°C for bacteria and at 25°C–27°C for fungi. The antimicrobial screening was conducted based on the method described previously (Handayani *et al.*, 2015a; 2017a; 2017b; 2018a; 2018b). The clear zones in the media were an indication of antimicrobial activities. The diameter of the inhibition zones was measured, and the standard deviations were also determined. The strain that showed inhibition zone >10 mm was selected for phytochemical test, microscopic examination, and molecular identification.

### Screening for Toxicity and cytotoxic activity

A preliminary study for toxicity activity was conducted with the BSLT method which as written in the previous research report (Handayani *et al.*, 2018a; 2018b; Meyer *et al.*, 1982). The present cytotoxic assay was performed by MTT assay using T47D (human ductal breast epithelial tumor) cell lines using four potential fungi. Fungal extracts were made in various concentrations of 1,000, 500, 250, 125, 62.5, and 31.25 ppm. Value Ln concentration is regressed against cell viability. Regression equations were then used to calculate the IC<sub>50</sub> value.

### Phytochemical test

The phytochemical examination was carried out for the EtOAc extract of symbiotic fungi that had the clear zone >10 mm as the standard methods described previously (Handayani *et al.*, 2019a; 2019b; Tiwari *et al.*, 2011).

### Microscopic examination

The potential fungi with cytotoxic activity were examined under a microscope with a magnification of ×2000. The specimen was put on object glass and dripped with lactophenol and mixed. The specimen was covered with cover glass. The morphology of the specimen was observed, and the result was compared with literature.

### Molecular identification

The procedure for DNA extraction was performed according to the modification method of Atashpaz *et al.* (2010). Four endophytic fungal strains with the highest antimicrobial and cytotoxic activities were identified using the internal transcribed spacer (ITS) region. The Polymerase Chain Reaction (PCR) reaction contained KOD FX Neo (Toyobo, Tokyo, Japan) with ITS1 and ITS4 primers, and the amplification was carried out using the method described by Ferrer *et al.* (2001).

The PCR products were sent to the first BASE, Malaysia, for sequencing. The sequences were subjected to pairwise sequence alignment using the BLAST (Basic Local Alignment Search Tool) program on NCBI (National Center for Biotechnology Information) and MycoBank database. The phylogenetic tree was constructed using the neighbor-joining

method, and p-distance model was set with a bootstrap value of 1,000 replication integrated into MEGA 7.0 software (Kumar *et al.*, 2016).

## RESULTS AND DISCUSSION

Nine fungal isolates (designated as Dc01 to Dc09) from sponge *Dactylosporgia* sp. were able to be isolated. The ethyl acetate extract of each fungal isolates was tested for antimicrobial activities. Four fungal isolates showed good antibacterial activities in various concentrations against *S. aureus* and *E. coli* (Table 1). Davis and Stout (1971) classified the strength of inhibition into four categories based on the diameter of inhibition as follows: very strong ( $\geq 20$  mm), strong (10–20 mm), medium (5–10 mm), and weak ( $\leq 5$  mm). The fungal extracts of Dc03, Dc04, and Dc08 in the concentration of 5% showed strong inhibition against *S. aureus* and *E. coli*. The isolates Dc03 and Dc04 were selected for further test against resistant bacteria. The isolate Dc04 showed the biggest inhibition zone for MRSA with a diameter of  $15.50 \pm 0.54$  mm. The potential fungi were next subjected to determine the phytochemical constituents and identification of species.

Cytotoxic activity screening results are listed in Fig. 1. If the  $LC_{50}$  value is below 1,000  $\mu\text{g/ml}$ , then the sample is classified as toxic, and if it is greater than 1,000  $\mu\text{g/ml}$ , it is classified as nontoxic (Meyer *et al.*, 1982). In this present study, all fungal extracts were toxic and, therefore, have the potential to produce cytotoxic compounds. The five fungal isolates with  $LC_{50}$  value below 80  $\mu\text{g/ml}$  were selected further to determine their active compounds and species identification.

Further testing on the cytotoxic activity was conducted by MTT assay using the T47D cell lines. The first step was the determination of the viability percentage of cancer cells in the culture medium after the addition of fungal extracts with a concentration of 100  $\mu\text{g/ml}$ . Data obtained were analyzed by a linear regression equation to obtain  $IC_{50}$  value. The  $IC_{50}$  extract value of Dc03, Dc04, Dc05, and Dc08 against T47D cell line difference was in the range of 225.75–1,760.98 ppm (Table 2). The potential fungal extracts did not show good  $IC_{50}$  value. It

might be due to the cytotoxic activity of fungal extracts selective to another human cancer cell lines.

The results of the screening of antimicrobial and cytotoxic activity showed that the isolates such as Dc03, Dc04, Dc05, and Dc08 had a high potential as antibacterial and cytotoxic agents. The chemical constituent analysis showed that most of the fungal extracts contained terpenoid, steroid, and flavonoid but did not contain alkaloid, phenol, and saponin (Table 3).

The morphological observations showed that isolate Dc03 was greenish-brown, the surface was centered to the middle, and the bottom was black with white flat edges (Fig. 2). The microscopic identification showed that this fungus had round conidia bulkhead along the conidiophores (Fig. 2). The examination of macroscopic and microscopic observation was concordance with literature that Dc03 was identified as *Cladosporium halotolerans* MN859971. This result was confirmed by molecular identification conducted at Indonesian Center for Biodiversity and Biotechnology. A BLAST search in NCBI-GenBank and MycoBank showed that isolate Dc03 had 100% identity with *C. halotolerans* MN859971. The phylogenetic tree showed that Dc03 was clustered together with *C. halotolerans* strain DTO127E8 (Fig. 3). The study about secondary metabolites from *C. halotolerans* is still limited. Pang *et al.* (2017) reported that *Cladosporisteroid* A, B, and C and *Cerevisterol* compounds isolated from *Cladosporium* sp. which was derived from marine sponge *Callyspongia* sp. showed antiviral and cytotoxic activities (Pang *et al.*, 2017).

The fungus Dc04 strain was brownish-green, the surface was stripped to the center, and the bottom was yellow-white and white flat edges (Fig. 2). In the microscopic observation, Dc04 had conidiophores that are not broad but branched, phialide, and with round conidia (Fig. 2). The isolate Dc04 was identified molecularly as *Penicillium citrinum* MN859968. On the phylogenetic tree, the isolate Dc04 resided in the same with *P. citrinum* strain ercha 16 (Fig. 3). The secondary metabolites isolated from the marine-derived fungi from the genus *Penicillium* produced various bioactive compounds with wide biological activities, such as

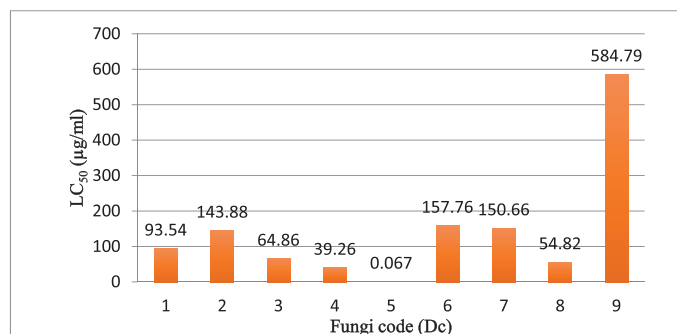
**Table 1.** Antimicrobial activity of EtOAc extracts of marine-derived fungi from *Dactylosporgia* sp.

Fungi code	Zone of inhibition (mm) $\pm$ standard deviation (SD)										
	<i>E. coli</i>			<i>S. aureus</i>			<i>C. albicans</i>			<i>MRSA</i>	<i>MDRPA</i>
	5%	3%	1%	5%	3%	1%	5%	3%	1%	5%	5%
Dc01	8.51 $\pm$ 0.40	6.43 $\pm$ 0.05	-	-	-	-	-	-	-	n	n
Dc02	-	-	-	-	-	-	-	-	-	n	n
Dc03	12.74 $\pm$ 0.45	11.28 $\pm$ 0.10	9.56 $\pm$ 0.36	11.84 $\pm$ 0.10	11.28 $\pm$ 0.10	9.39 $\pm$ 0.14	-	-	-	10.88 $\pm$ 1.25	9.42 $\pm$ 1.59
Dc04	15.50 $\pm$ 0.30	14.21 $\pm$ 0.25	11.08 $\pm$ 0.30	16.14 $\pm$ 0.75	12.75 $\pm$ 0.27	10.30 $\pm$ 0.24	-	-	-	15.50 $\pm$ 0.54	8.33 $\pm$ 0.29
Dc05	10.41 $\pm$ 0.42	8.01 $\pm$ 0.43	6.21 $\pm$ 0.14	6.37 $\pm$ 0.42	-	-	-	-	-	n	n
Dc06	6.53 $\pm$ 0.39	-	-	-	-	-	-	-	-	n	n
Dc07	6.50 $\pm$ 0.23	-	-	8.70 $\pm$ 0.35	6.42 $\pm$ 0.28	-	-	-	-	n	n
Dc08	12.31 $\pm$ 0.54	10.55 $\pm$ 0.40	7.53 $\pm$ 0.29	14.25 $\pm$ 0.10	9.46 $\pm$ 0.29	7.19 $\pm$ 0.13	-	-	-	n	n
Dc09	6.15 $\pm$ 0.11	-	-	8.61 $\pm$ 0.21	7.10 $\pm$ 0.05	-	-	-	-	n	n
Positive control		22.15			22.35			22.15		24	8.75
Negative control		-			-			-		-	-

- = no inhibition

n = not tested





**Figure 1.** The cytotoxic activity of EtOAc extracts of marine-derived fungi from *Dactylosporgia* sp.

**Table 2.** The IC<sub>50</sub> value of selected fungal extracts on T47D cancer cell line.

No.	Code of Extract	The IC <sub>50</sub> value of T47D cell line (ppm)
1	Dc03	225.75
2	Dc04	640.12
3	Dc05	1,760.98
4	Dc08	456.75
5	Doxorubicin	10.05

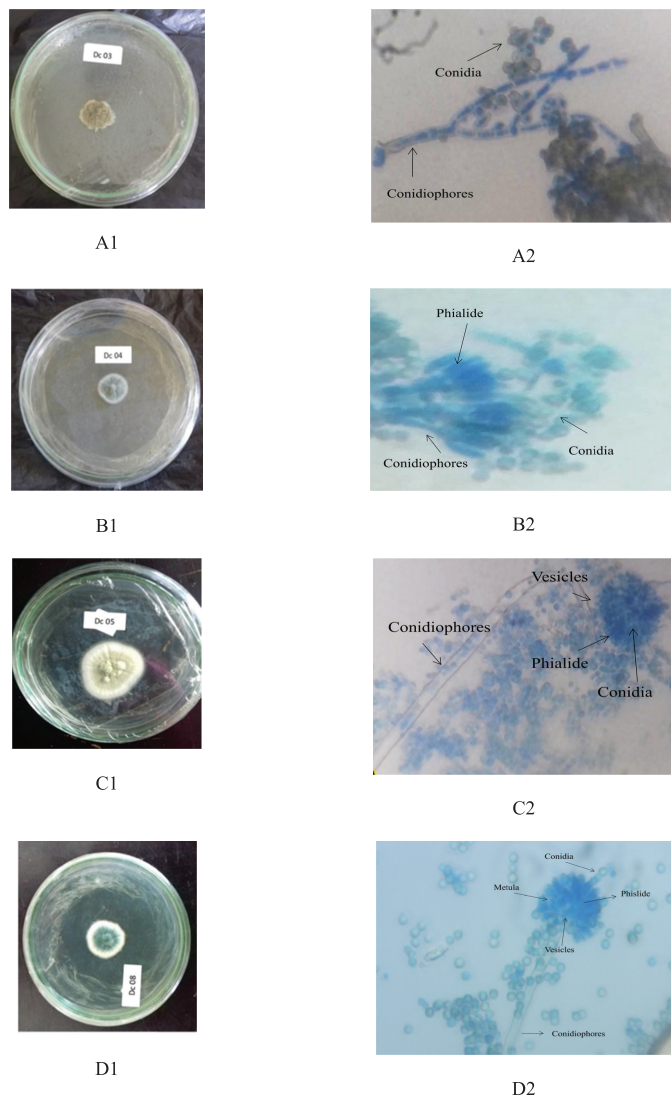
**Table 3.** The chemical constituents of EtOAc extracts of marine-derived fungi from *Dactylosporgia* sp.

Fungi code	Chemical constituents					
	Alkaloid	Flavonoid	Terpenoid	Steroid	Phenolic	Saponin
Dc01	-	-	-	+	-	-
Dc03	-	+	-	+	-	-
Dc04	-	+	+	-	-	-
Dc05	-	-	+	+	-	-
Dc08	-	-	+	+	-	-

Note: - = no reaction

antifouling polyketides (Bao *et al.*, 2013), antibacterial communal A (Wang *et al.*, 2012), cytotoxic penicoline (Shao *et al.*, 2010), and many others. Some bioactive compounds from *P. citrinum* that had been reported included citrinin (Subramani *et al.*, 2013), isocyclocitrinol A and 22-acetylisocyclocitrinol A (Amagata *et al.*, 2003), and  $\gamma$ -sitosterol, stigmasterol, pentadecanoic acid, and 14-methyl-methyl ester-17-Pentatriacontene (Binh *et al.*, 2018). The genera of *Penicillium* are not only marine borne but also soil borne as well. Even though their terrestrial counterparts have been the subject of chemical screening for at least five decades, marine-derived *Penicillium* sp. continues to yield a broad of new secondary metabolites.

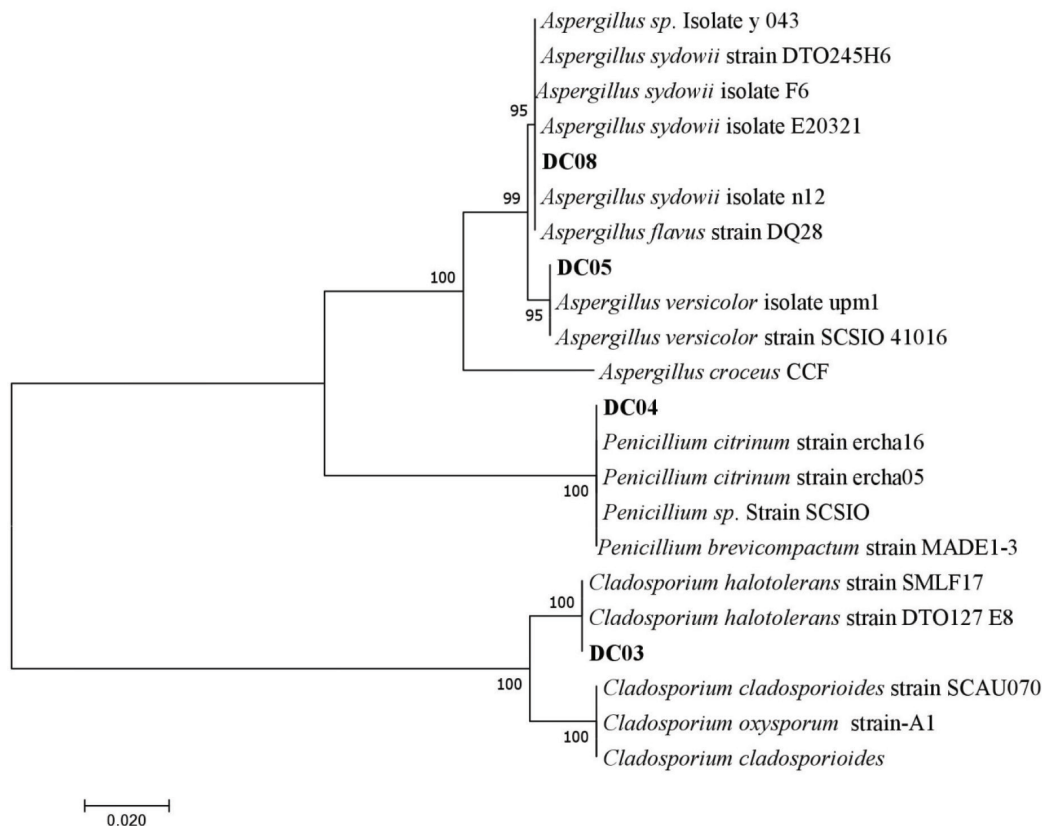
The next potential fungi were Dc05 which had yellowish-green, hairy surface, white, and flat edges' colony (Fig. 2). Dc05 had round conidia, conidiophores, phialide, and vesicles (Fig. 2). The macroscopic and microscopic determination allowed Dc05 as *Aspergillus versicolor* MN859970. Molecular identification showed that Dc05 had an identity 100% with *A. versicolor* strain upm1 on BLAST analysis (Fig. 3). The isolate Dc05 had the highest cytotoxic activity with the LC<sub>50</sub> value of 0.067  $\mu$ g/ml. Many secondary metabolites have been isolated from *A. versicolor* with various biological activities, such as



**Figure 2.** The macroscopic (left side) and microscopic (right side) observations of the fungi. A1 and A2 are the isolate Dc03 (*C. halotolerans* MN859971), B1 and B2 are the isolate Dc04 (*P. citrinum* MN859968), C1 and C3 are the isolate Dc05 (*A. versicolor* MN859970), and D1 and D2 are the isolate Dc08 (*A. sydowii* MN859969).

5-methoxydihydrosterigmatocystin as antimicrobial agent (Song *et al.*, 2014), aspergoterpenins A-B with antimicrobial and cytotoxic activity (Guo *et al.*, 2018), 1'-O-ethyl-6,8-di-O-methylaverantin as antibacterial agent (Zhang *et al.*, 2012), and novel anthraquinone [2-(dimethoxymethyl)-1-hydroxyanthracene-9,10-dione] which exhibited strong inhibitory activities against MRSA ATCC43300 and MRSA CGMCC 1.12409 (with MIC (minimum inhibitory concentration) values of 3.9 and 7.8  $\mu$ g/ml, respectively) and moderate activities against tested strains of *Vibrio* (with MIC values ranging from 15.6 to 62.5  $\mu$ g/ml) (Wang *et al.*, 2018). This fungus can also be found in a plant, soil, marine habitat, and air (Ebada *et al.*, 2018).

The macroscopic characterization showed that the isolate Dc08 colony was solid green with hairy surface and white-flat edges (Fig. 2). This fungus had round conidia, conidiophores, vesicles, phialide, and metula (Fig. 2). Molecular identification



**Figure 3.** Neighbor-joining phylogenetic tree of potential fungal isolates from *Dactylosporgia* sp.

showed that Dc08 had 100% identity with *Aspergillus sydowii* strain E20321 ITS and identified as *A. sydowii* MN859970 (Fig. 3). Gao *et al.* (2017) reported about bioactive compounds from *A. sydowii*, a fungal strain obtained from the Marine Culture Collection of China. Lumichrome, N-acetyltyramine, 3-(2-hydroxypropyl)-4-(hexa-2E,4E-dien-6-yl)furan-2(5H)-one, 1,4-dioxa-9,12-diazacyclohexadecane-5,8,13,16-tetraone, and cycloerodiol were succeed isolated from the EtOAc extract of this fungal and displayed moderate antibacterial activity with an MIC value between 6.3 and 25.0  $\mu$ M.

The bioactivities of secondary metabolites from marine fungi reveal interesting pharmacological activities, and the fungi from the genera *Penicillium* and *Aspergillus* were the major object in this field. Most of the published work on secondary metabolites of marine fungi has focused on these genera due to the ability of these fungi to produce the new compounds (Imhoff, 2016).

## CONCLUSION

Marine-derived fungi have already provided a variety of pharmacologically active novel secondary metabolites and thus represent a valuable resource for structurally unique and interesting biological properties. In this study, we had succeeded in the isolation of nine fungi-derived marine sponge *Dactylosporgia* sp. and screened for their antimicrobial and cytotoxic activities. In further research, it is challenging to isolate the bioactive compounds from the potential marine-derived fungi, especially for *P. citrinum* Dc04 for the discovery of new drug candidates.

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## CONFLICT OF INTERESTS

Authors declare that they do not have any conflicts of interest.

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