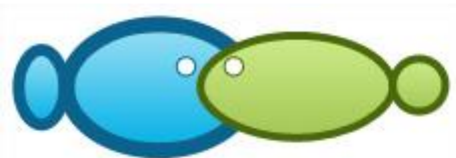

Antimicrobial and cytotoxic activities screening of fungal secondary metabolites isolated from marine sponge *Callyspongia* sp.

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Abstract. Secondary metabolites from marine sponge-derived fungi repeatedly showed potential bioactive activity against several diseases. In this study, screening for antibacterial and cytotoxic isolated fungi from marine sponge *Callyspongia sp.* have been performed. A common media for culturing fungi, sodium dextrose agar (SDA), was used. For fungal isolates the streak plate method was used. Each pure fungal isolate was cultivated on rice media under room temperature for 4-8 weeks then extracted with ethyl acetate. Thirteen pure fungal isolates were successfully obtained from *Callyspongia* sp. Ethyl acetate extract of all isolated fungi was tested for antimicrobial activity against pathogenic microbes using the disk diffusion method and cytotoxic activity using brine shrimp lethality test (BSLT). Of thirteen fungal isolates, only Cas 02 showed antimicrobial activity against *Staphylococcus aureus* (SA) ATCC 2592 and *Escherichia coli* (EC) ATCC 25922 with a diameter of inhibition zone of 12.75 mm and 17.16 mm respectively. The cytotoxic activity results showed that only isolate Cas03 had a potential activity with LC_{50} < 30 μg mL^{-1} and four fungal strains (Cas02, Cas06, Cas07, and Cas09) had a moderate activity with LC_{50} < 100 μg mL^{-1}. Molecular identification showed that Cas02 was *Aspergillus unguis*, Cas03 was *Penicillium citrinum*, Cas06 and Cas07 were *A. flavus*, and Cas09 was *A. austroafricanus.*

Key Words: marine sponge-derived fungi, *Aspergillus unguis*, *Penicillium citrinum*, *Aspergillus flavus*, *Aspergillus austroafricanus*.

Introduction. The discovery of new antibiotics and new anticancer treatments are strictly important to resolve drug resistance (Mabona et al 2013; Balouiri et al 2016; Cragg & Newman 2018). New drug molecules are mostly discovered from natural sources (Newman & Cragg 2016). Plants, marine biota, bacteria, fungi are the major source where potential new drug molecules are derived from (Strobel & Daisy 2003; Berdy 2005; Runyoro et al 2006; Gomes et al 2012; Nazzaro et al 2013). This kind of investigations makes the exploration of natural products become an interesting field (Balouiri et al 2016).

Marine sponge-derived fungi are one of the important natural resources for producing unique chemical structures with potential bioactive fetaures (Wiese et al 2011; Bovio et al 2019; Pang et al 2019). Important alkaloids, polyketides, terpenoids have been produced by many fungi that are associated with the marine sponge (Ma et al 2016; Elissawy et al 2017; Lei et al 2019; Pang et al 2019). New alkaloids produced by *Penicillium* sp. SCSO41015 that derived from marine sponge *Callyspongia* sp*.* displayed potent cytotoxic and antibacterial activities (Pang et al 2019). Lei Hui et al (2019) reported a new polyketide with potent cytotoxic activity isolated from a marine sponge-derived fungus *Pestalotiopsis heterocornis* XWS03F09.

Marine sponge *Callyspongia* sp*.* originated from Mandeh island, west Sumatra-Indonesia has been explored in the present study. Our team, recently studied, some of the sponges that originated from Mandeh island including *Haliclona fascigera*, *Neopetrosia chaliniformis*, and *Acanthrongylophora ingens*. Some bioactive secondary metabolites producing fungi were successfully isolated from these sponges (Handayani et

al 2015, 2018; Handayani & Aminah 2017; Handayani & Artasasta 2017; Aminah et al 2020).

Material and Method

Sponge identification. Marine sponge *Callyspongia* sp. was harvested by scuba diving, from Mandeh island, West Sumatra-Indonesia. Sponge taxonomy identification was conducted by Dr. Nicole J. De Voogd, at the Natural Biodiversity Center, Netherland. The sponge was identified as *Callyspongia* sp.

Symbiotic fungal isolation and extraction of secondary metabolites. Isolation and cultivation of symbiotic fungi were carried out according to Kjer et al (2010). The fungi were cultivated on rice then extracted with ethyl acetate (EtOAc). The extracts were collected by evaporating the solvent. Furthermore, the extracts were used for testing their antimicrobial and cytotoxic activities including the phytochemical tests.

Screening for antimicrobial activity. An antimicrobial activity test was conducted using the agar disk diffusion method. In this study, the fungal extracts were tested against *Staphylococcus aureus* (SA) ATCC25923, *Escherichia coli* (EC) ATCC25922, *Candida albicans* (CA), and clinical MRSA isolate obtained from Cipto Mangunkusumo Hospital, Jakarta, Indonesia. Ten microliters of 5% extract were pipetted onto the 6 mm sterile paper disks until saturation. The saturated paper disks were placed onto nutrient agar (NA) for antibacterial test and sabouraud dextrose agar (SDA) for an antifungal test. The dimetil sulfoxide (DMSO) was used as a negative control. Chloramphenicol and nystatin were used as positive controls. The diameter inhibition zone was determined after incubation at room temperature for 24 h (Balouiri et al 2016; Zabidi et al 2020).

Screening for cytotoxic activity

Brine shrimp lethality test (BSLT). 1000, 100, and 10 μg mL^{-1} of fungal extracts were serially diluted in DMSO. In this study, ten nauplii were exposed to different concentrations of fungal extract in the test tubes containing 5000 μL of seawater. The LC_{50} was determined by using probit analysis (Meyer et al 1982).

MTT assay. Breast cancer cells T47D were used for cytotoxic activity of potential fungal extracts. The IC_{50} value was determined by using MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent. One thousand, 500, 250, 125, 62.5, and 31.25 μg/mL of fungal extracts were added into the 96-well plates which already contained confluent cells. After 24h of incubation, the media were discarded and the cells were washed with phosphate-buffered saline (PBS). Furthermore, the MTT reagent was again added to the well and incubated for 4h. DMSO was used to stop the reaction. The absorbance was determined by using an ELISA reader (Bio-red). The percentage of viable cells was converted to the IC_{50} value (Artasasta et al. 2017; Handayani et al. 2018).

Fungal Identification

Macroscopic and microscopic identification. The morphology of the fungi was macroscopically studied by observing the features of the colony including the color, shape, size, and hyphae. The small portion of the fungal mycelium was mounted on the glass slide, stained with lactophenol cotton blue, and identified microscopically using a compound microscope with a digital camera. The conidiophore, vesicle, and conidia were observed (Afzal et al 2013).

Molecular identification. Molecular identification was conducted using an internal transcribed spacer (ITS*)* primer. The DNA extraction including the polymerase chain reaction (PCR) was carried out by following the protocol provided by Saitoh et al (2006). The sequencing of the PCR product was performed at First Base, Malaysia. The sequences were subjected to the BLAST program at the National Center for Biotechnology Information NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGETYPE

=BlastSearch&LINK_LOC=blasthome), pairwise sequence alignment at Mycobank, and the barcode of life data system (BOLD). The highest identity indicates that the species being identified. The phylogenetic tree of the identified isolates was performed using MEGA 7.0 software (Saitoh et al 2006; Kumar et al 2016).

Phytochemical test. The phytochemical test was conducted by following the standard protocol of Harborne (1973).
Alkaloids. Ethyl acetate extract was spotted onto the G60 F254 silica plate, then eluted with n-hexane : ethyl acetate eluent (1:4). To make spots visible, the spots were treated with Dragendorff's reagent. If the color turned orange, the extract contained alkaloids.

Terpenoids and steroids. Ethyl acetate extract was spotted on the G60 F254 silica plate, then eluted with n-hexane : ethyl acetate eluent (1:4). The Lieberman Burdchart's was dropped on a cotton bud and swapped equally and thinly. If the reaction was not spontaneous, heating was needed. Positive extracts contained terpenoids if they formed pink color and positive steroids formed blue and green color.

Phenolic compounds. Ethyl acetate extract was spotted on the G60 F254 silica plate, then eluted with n-hexane : ethyl acetate eluent (1:4). The $FeCl_3$ reagent swapped on the plate evenly and thinly using a cotton bud. Positive extracts contained phenolic if they formed purple, red, or pink.

Flavonoid. Ethyl acetate extract was spotted on the G60 F254 silica plate, then eluted with n-hexane : ethyl acetate eluent (1:4). The Citroborat reagent was applied on the plate equally and thinly using a cotton bud. Positive extracts contained flavonoids if they formed green color.

Results. Thirteen fungal isolates were obtained from marine sponge *Callyspongia* sp. Isolate Cas02 showed potential antibacterial activity against SA and EC with the diameter of inhibition zone of 12.75 and 17.15 mm, respectively (Table 1). However, it showed no activity against CA. Other isolates generally showed moderate activity against SA, EC, and CA. Extract of isolate Cas02 can be categorized as having broad-spectrum antimicrobial activity as it displayed the ability to inhibit both Gram-negative and Gram-positive bacteria (Balouiri et al 2016; Handayani et al 2019). Isolate Cas02 also showed inhibition activity against MRSA with the diameter of the inhibition zone of 13.75±2.09 mm.

Table 1

Antimicrobial activity of fungi isolates from marine sponge *Callyspongia* sp.

No.	*Sample code*	*Inhibition zone diameter (mm) ± Deviation standard (SD)*		
		SA	*EC*	*CA*
1	Cas 01	-	-	8.41 ± 1.37
2	Cas 02	12.75±0.66	17.16±2.12	-
3	Cas 03	-	7.91±1.77	7.29±1.75
4	Cas 04	8.33±1.12	8.12±1.18	8.16±1.37
5	Cas 05	7.31±0.31	7.79±1.36	6.45±0.79
6	Cas 06	6.58±0.38	7.33±0.57	7.87±1.96
7	Cas 07	7.45±0.73	7.00±0.00	7.91±1.58
8	Cas 08	8.25±0.25	8.00±1.00	6.04±0.19
9	Cas 09	6.83±0.28	7.58±1.01	7.16±0.28
10	Cas 10	-	6.50±0.50	7.58±1.62
11	Cas 11	7.58±1.01	7.00±0.00	7.20±0.83
12	Cas 12	-	7.16±0.76	-
13	Cas 13	9.74±0.21	8.95±0.93	7.16±0.28

The result of cytotoxic activity is showed in Figure 1. The brine shrimp lethality test was used for determining the cytotoxic activity of fungi isolated from marine sponge *Callyspongia* sp*.* In thepresent study, only isolate Cas03 had a rather high activity with LC_{50} < 30 μg mL^{-1}, and four other fungal strains (Cas02, Cas06, Cas07, and Cas09) had a moderate activity with LC_{50} < 100 μg mL^{-1}. However, these isolates showed low activity against breast cancer cells T47D with IC_{50} which ranged from 365 to1,500 μg mL^{-1}.

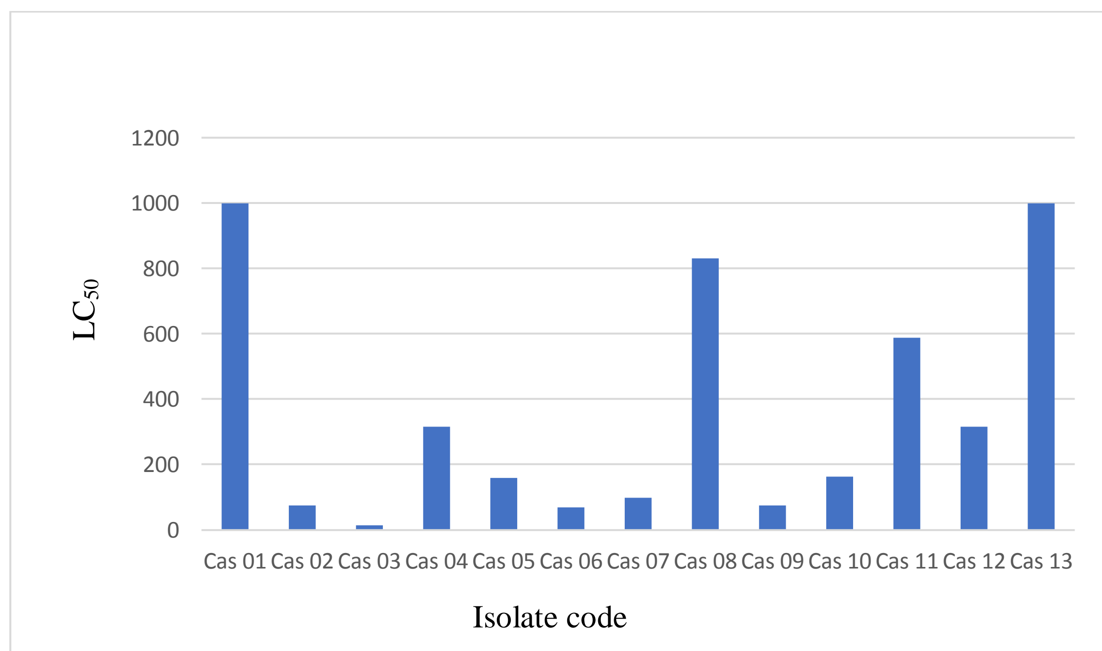

Figure 1. Cytotoxic activity result of fungal isolates from marine sponge *Callyspongia* sp.

Figure 2 shows the macroscopic and microscopic observations of isolates Cas02, Cas03, Cas06, Cas07, and Cas09. The isolate Cas 02 had white mycelium with a diameter of colonies after 5 days of ±2-3 cm (A1). Under microscopic observation, this isolate was observed to have blue light conidiophores. The asexual spores of this fungus were similar to the *Aspergillus* genus (A2). The isolate Cas03 was observed macroscopically to have green white mycelia with a diameter of colonies after 5 days of ±1-3 cm (B1). This strain was detected under microscopy and it showed blue conidiophore. Asexual spores of this fungal were similar to the *Penicillium* genus (B2). The isolate Cas06 shad predominantly dark green mycelia with white edges. The diameter of the colonies after 5 days was 6-8 cm (C1). Asexual spores of this isolate were similar to the *Aspergillus* genus (C2). The colonies of isolate Cas07 were green with white edges and a cotton-like surface. Diameters of the colonies after 5 days were 6-8 cm (D1). Asexual spores of this isolate were similar to the *Aspergillus* genus (D2). The color mycelia of isolate Cas09 was white with a diameter of colonies after 5 days of 2-3 cm (E1). Asexual spores of this strain were similar to the *Aspergillus* genus (E2).

The results of molecular identification using the ITS gene are presented in Table 2. Isolate Cas02 was identified as *Aspergillus unguis*, Cas03 as *Penicillium citrinum*, Cas06 and Cas07 as *A. oryzae*, and Cas09 as *Aspergillus austroafricanus*. All of these fungi belong to the Trichocomaceae family. The evolutionary history (Figure 3) was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al 2011).

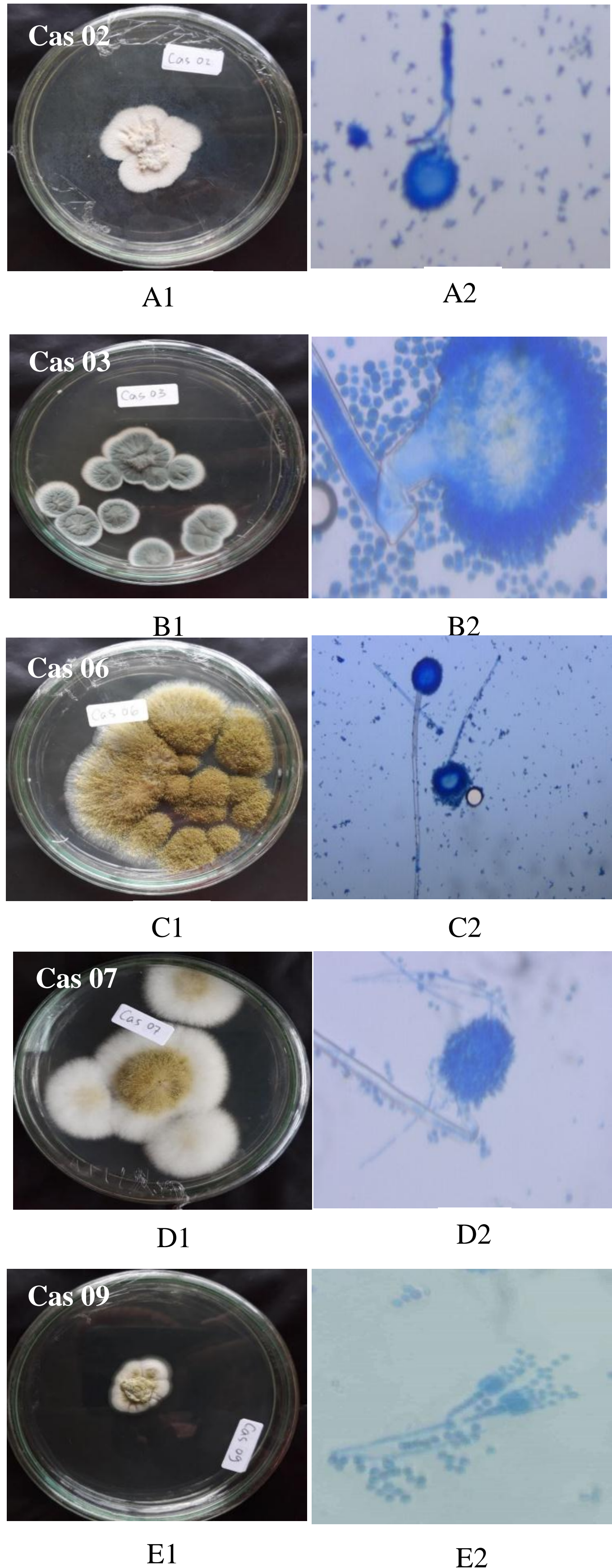

Figure 2. Macroscopic (left side) and microscopic (right side) observation of fungal isolates from marine sponge *Callsypongia* sp.

Table 2
Molecular identification result of isolates Cas02, Cas03, Cas06, Cas07, and Cas09

Isolate code	*Mycobank*	*Identity*	*NCBI*	*Identity*	*BOLD*	*Identity*
Cas02	*Aspergillus unguis*	100%	*A. unguis*	100%	*A. unguis*	100%
Cas03	*Penicillium citrinum*	100%	*P. citrinum*	100%	*P. citrinum*	100%
Cas06	*A. oryzae*	100%	*A. oryzae*	100%	*A. oryzae*	100%
Cas07	*A. oryzae*	100%	*A. oryzae*	100%	*AA. oryzae*	100%
Cas09	*A. austroafricanus*	100%	*A. austroafricanus*	100%	*A. austroafricanus*	100%

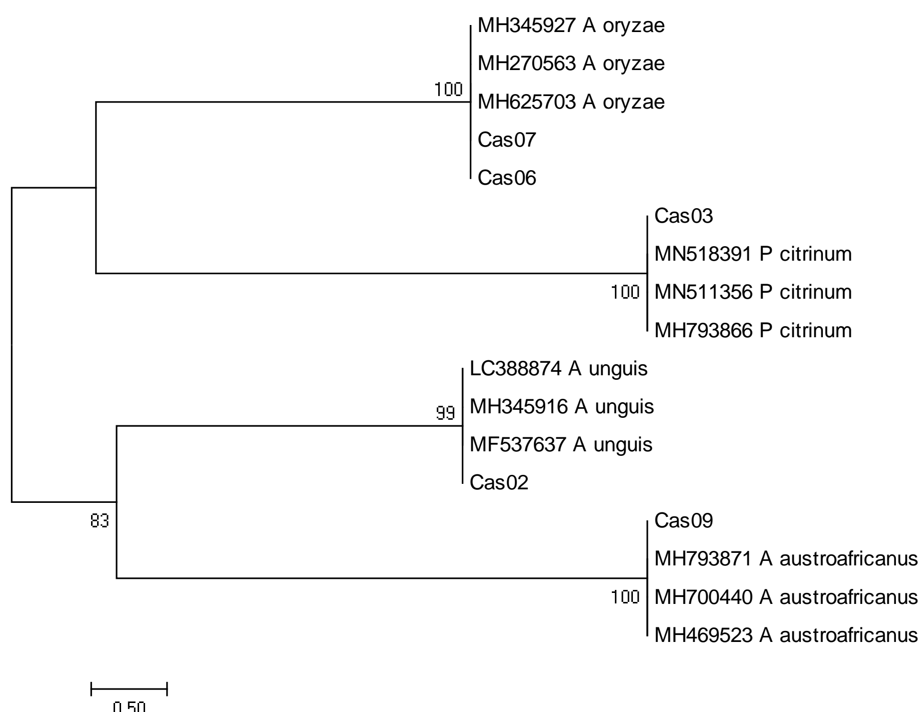

Figure 3. The phylogenetic tree of ITS sequences of Cas02, Cas03, Cas06, Cas07, and Cas09 was inferred using the Neighbor-Joining method.

Antimicrobial and cytotoxic activities of marine sponge-derived fungi isolated from *Callyspongia* sp. are thought to be caused by the secondary metabolites. Therefore, the phytochemical test was performed to study whether the secondary metabolites produced by these fungi had the above features. *A. unguis* strain Cas02 and *P. citrinum* strain Cas03 showed the most potent antimicrobial and cytotoxic activities compared to other strains. The phytochemical test result showed that strain Cas02 was positive containing steroid compounds. Strain Cas03 showed negative results for all secondary metabolite groups. Nonetheless, strain Cas03 probably contains other secondary metabolite types of fungi such as polyketide, peptides, shikimate, glycosides, isoprenoids, and non-isoprenoids (Suryanarayanan 2012; Hasan et al 2015; Ruiz-Torres et al 2017).

Hamed et al (2018) showed that different extracts from *A. unguis* SPMD-EGY exhibited antimicrobial and antioxidant activities. Recently, Wang et al (2019) isolated citrinin monomer and dimer derivatives from *P. citrinum* NLG-SO1-P1 obtained from deep-sea. *P. citrinum* isolated from mangrove *Bruguiera sexangular* produced new benzopyran derivative, (2R’,4R’)-3-4-dihydro-5methoxy-2methyl-2H-1-benzopyran-4-ol. Other compounds 6-methylcurvulinicacid, (+)-formylanserinone B, 3,5-dimethyl-8-methoxy-3,4-dihydro-1H-isochromen-6-ol, and quinolactacide were successfully isolated

from *P. citrinum* which were associated with brown alga *Padina* sp. These compounds displayed moderate cytotoxic activity against murine erythrocyte carcinoma (Zheng et al 2015; Smetanina et al 2016).

Secondary metabolites asporyzin C produced from marine *A. oryzae* showed antibacterial activity against *E. coli* (Qiao et al 2010). *Aspergillus austroafricanus* isolated from aquatic plant *Eichhornia crassipes* fermented on solid rice medium produced xanthone dimer austradixanthone and the sesquiterpene (+)-austrosene, with weak cytotoxicity against the murine lymphoma L5178Y cell line, but actively prevent the growth of *S. aureus* (ATCC 80099) (Ebrahim et al 2016).

Meenupriya & Thangaraj (2010) found that *A. flavus* isolated from *Callyspongia* spp*.* exhibited antibacterial activity against *E. coli* and *S. aureus* (Meenupriya & Thangaraj 2010)*. Drechslera hawaiiensis* isolated from *Callyspongia aerizusa* collected from the Sea of Bali, Indonesia, produced novel spiciferone derivatives, as reported by Edrada et al (2000). Almeida et al (2012) reported that *Stachylidium* sp. isolated from *Callyspongia* cf. C. flammea produced novel phthalimidine derivatives marilines A1 and A2. These findings suggest that *Callyspongia* obtained from different habitats have different associated fungi that produced different bioactive compounds. These compounds can further be scaled up for large biomass production and stable formulation as novel drugs (Almeida et al 2012).

Conclusions. Marine sponge-derived fungal *A. unguis* strain Cas02 and *P. citrinum* strain Cas03 isolated from *Callyspongia* sp. showed potential antimicrobial and cytotoxic activities, respectively. Further study about these fungi is needed to obtain more information about their bioactive compounds that can represent an important role as antimicrobial and cytotoxic agents.

Acknowledgements. Acknowledgment is addressed to BOPTN of Andalas University, Padang, Indonesia, in the project of "Penenelitian Dasar Unggulan Klaster Riset-Publikasi Guru Besar Universitas Andalas (PDU-KRP1GB-Unand)", No. T/3/UN.16.17/PT.01.03/KO-PDU-KRP1GB/2020. We are also grateful to Dr. Nicole J. De Voogd, Natural Biodiversity Center, the Netherlands for the identification of the marine sponge.

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Received: 02 December 2020. Accepted: 11 February 2021. Published online: 18 February 2021.
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How to cite this article:
Handayani D., Artasasta M. A., Mutia D., Atikah N., Rustini, Tallei T. E., 2021 Antimicrobial and cytotoxic activities screening of fungal secondary metabolites isolated from marine sponge *Callyspongia* sp. AACL Bioflux 14(1):249-258.