BIOACTIVE COMPOUNDS FROM A MARINE SPONGE DERIVED FUNGUS *Penicillium oxalicum* WR3

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ABSTRACT

Chromatographic workup on ethyl acetate extract of fungus culture, *Penicillium oxalicum* WR3, isolated from the marine sponge *Haliclona fascigera*, collected from Mandeh Island, south coast of West Sumatera, Indonesia, resulted in the isolation of two known fungal metabolites, culvularin (1) and sydowinin B (2). These compounds were evaluated for the antibacterial towards *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA), as well as cytotoxic activities against Hela, T47D, and WiDR cell lines. Based on the results of the antibacterial assay, both compounds revealed relatively weak inhibition towards both tested bacterial strains when treated at a concentration of 125 µg/disc and 500 µg/disc, respectively. Furthermore, compound 1 showed mild cytotoxicity against the human cancer cell lines, T47D and HeLa, with IC₅₀ values of 142.66 ppm and 164.88 ppm, respectively.

Keywords: *Penicillium oxalicum*, *Haliclona fascigera*, Antibacterial, Cytotoxic, Culvularin, Sydowinin B.

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INTRODUCTION

The sponge is one of the abundant marine organisms in Indonesia. Based on the Snellius-II expedition, there are 830 types of marine sponges found in western Indonesia.¹ The sponge is also known as a very fertile host for a variety of microorganisms. Microbial symbionts can be either bacterial or fungal and contribute over 40-70 % of the animal biomass and produce secondary metabolites.^{2,3} In particular, marine sponge-derived fungi have attracted attention for their production of structurally unique molecules with intriguing bioactivities.⁴ Examples include the antibacterial bisabolane-type sesquiterpenoids from sponge-associated fungus, *Aspergillus* sp., as well as the novel and potent antimycobacterial compounds, trichoderins A, A1, and B, isolated from marine sponge-derived fungus, *Trichoderma* sp.⁵ Recently, we have examined several fungi from marine sponges and mangrove plants from West Sumatra, Indonesia, which show their ability to produce antibacterial and cytotoxic compounds, including *Aspergillus nomius* NC06, *Cocliobolus geniculatus* WR12 and *Diaporthe amygdali* SgKB4.⁶⁻⁸

As part of our continuous research for bioactive molecules from the marine sponge-derived fungi, we investigate *Penicillium oxalicum* WR3, a fungus associated with the marine sponge *Haliclona fascigera*. The sponge, *H. fascigera*, was found in Mandeh Island, south coast of West Sumatera, Indonesia. Preliminary studies on ethyl acetate extract of *P. oxalicum* WR3 showed its potential for antibacterial and cytotoxic activities.^{9,10} In the current study, we present details of the isolation, and structure identification of two metabolites, culvularin (1) and sydowinin B (2) isolated from ethyl acetate extract of *P. oxalicum* WR3, as well as, their antibacterial and cytotoxic activities.

EXPERIMENTAL

General Experimental Procedures

Organic solvents are distilled before being used for extraction and chromatography. Solvents for spectrophotometry were obtained from Merck®. Fungal growth media *Sabouraud Dextrose Agar* (SDA) (Merck®), Bacteria growth media *Nutrient Agar* (NA) (Merck®), a disk of chloramphenicol (BD BBL™) and a disk of nystatin (Oxoid®). Fractions were monitored on TLC silica gel 60 F₂₅₄nm (Merck®) and visualized under UV_{λ254nm} and UV_{λ365nm}. Open column chromatography was carried out on Silica gel 60 (0.040 – 0.005 mm) Merck® (Darmstadt, Germany) and Gel filtration on sephadex™LH-20 (GE healthcare®). Melting points were measured using the Innotech® *Melting Point Apparatus*. A Perkin-Elmer Spectrum One FT-IR instrument used for the experimental IR spectrum. The Ultraviolet spectrum was recorded on Shimadzu® ultraviolet-visible Pharmaspec 1700 spectrometer. 1D and 2D NMR spectra were recorded on Bruker AVANCE DMX 600 and 300 NMR spectrometers. The chemical shifts were referred to residual solvent signals at δ_H 2.50 (DMSO-*d*₆) for ¹H and δ_C 39.5 (DMSO-*d*₆) for ¹³C. Mass spectrum (ESI) was measured with a Finnigan LCQ Deca mass spectrometer and the HRESIMS spectrum was recorded with an FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer.

Fungal Material

The marine-derived fungus *P. oxalicum* WR3 was isolated from marine sponge *H. fascigera*. The marine sponge was collected from Mandeh Island, South Coast of West Sumatera, Indonesia. The fungus was grown in *Sabouraud Dextrose Agar* (SDA) media and incubated at 25° C for 4-7 days.¹⁰

Identification of Fungal Cultures

Fungal cultures were identified according to a molecular biology protocol by DNA amplification and sequencing of the ITS region as described previously.¹⁰ The fungal strain was identified as *Penicillium oxalicum*. A voucher strain (strain designation WR3) is kept in the Laboratory of Sumatra Biota/Faculty of Pharmacy, Andalas University, Padang, Indonesia.

Cultivation

The fungus was grown in twenty Erlenmeyer flasks (1 L each) containing 100 g of rice and 110 mL of distilled water at room temperature (25°C) for 40 days.¹¹

Extraction and Fractionation

The secondary metabolites which are produced by *P. oxalicum* WR3 were extracted with EtOAc. The EtOAc extract was then evaporated with a rotary evaporator and partitioned between n-hexane and 90% MeOH. The 90% MeOH fraction (5.7 g) was chromatographed on Sephadex LH-20 column with 100% MeOH as a solvent, to yield five subtraction. Subfraction IV (3.9 g) was further chromatographed over silica gel using *n*-hexane: EtOAc and EtOAc: MeOH as solvent systems. Based on detection by TLC using EtOAc: DCM (20:80) as a solvent system, collected fractions were combined. Two fractions were recrystallized, to afford compounds **1** (1124.5 mg) and **2** (164.2 mg).

MIC Assay Test for Antibacterial Activity

For the MIC (Minimum Inhibitory Concentration) assay of antibacterial activity, the antibacterial activity of compounds **1** and **2** was tested against *S. aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) using the agar diffusion method. DMSO and chloramphenicol (30 µg/disc) were used as a negative and positive control, respectively. The paper disc placed on the surface of *Nutrient Agar* medium containing 10⁵ of tested bacteria and incubated at 37° for 24 hours. The lowest concentration contained a clear zone around the paper disc was described as a minimum inhibitory concentration (MIC) value.⁶

MTT Assay Test for Cytotoxic Activity

The isolated compounds were tested on HeLa (cervix cell line), WiDr (colon adenocarcinoma cell line), T47D (human ductal breast epithelial tumor cell line) and Vero (normal cell line) using MTT assay¹². All compounds were tested in the Laboratory of Parasitology, Gadjah Mada University.

RESULTS AND DISCUSSION

Based on 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 WR3 isolate had a maximum identity of 99 % with *P. oxalicum* strain 114-2. According to Meusemann, *et al.* (2010)¹³, microorganisms with differences less than 2% or similarity more than 97% of 18S rRNA, could be concluded as identical species.

The phylogenetic tree constructed using a neighbor-joining method with a bootstrap value of 1000. The analysis results showed that tree formation consists of one clade. The clade was a group of *Penicillium*. The WR3 isolate gave 100% identity to *P. oxalicum*, thus confirmed the identification result (Fig.-1).

Fig.-1: The Phylogenetic Tree Using the Neighbor-joining Method of the 18S rRNA Gene Sequence From the Fungi isolate WR3 derived Marine Sponge *Haliclona fascigera* and Some Strain of *Penicillium*. The Scale Bar indicates a 0.05 substitution Nucleotide Position

Compound **1** (1124.5 mg) was isolated as colorless crystals, mp 203.3~203.9°C. The HRESIMS spectrum of **1** showed a prominent pseudomolecular ion peak at m/z 293.1384 $[M + H]^+$, attributed to the molecular formula $C_{16}H_{20}O_5$, corresponding to seven degrees of unsaturation. UV absorptions with λ_{max} in methanol at 302.8 and 271.1 nm were indicative of aromatic functionality with extended conjugation. The IR spectrum of **1** showed absorption bands for OH group at 3536.5 and 3269.5 cm^{-1} ; CH_2 at 2934.6 and 1691.1 cm^{-1} ; $C=C$ aromatic at 1598.7 cm^{-1} ; and $C-C$ at 999.3 cm^{-1} . The 1H NMR data of **1** (Table-1) displayed signals of two aromatic protons in *meta*-position, six sets of methylene, one oxygenated methine and, one methyl group. Moreover, ^{13}C NMR revealed the signals of two carbonyls and four sp^2 carbons, of which two were oxygenated ones. Analysis of 2D NMR in combination with HRESIMS data of **1**, showed a good agreement with those reported in the literature for curvularin¹⁴. Hence, the structure of **1** was concluded to be identical

to culvularin (Fig.-2). Culvularin, a macrocyclic lactone, was repeatedly reported from numerous fungal species belonging to the genus *Penicillium*,^{15,16} *Curvularia*,¹¹ and *Alternaria*.¹⁷ Compound **2** (164.2 mg) was obtained as yellow needle crystals, mp 155.5 ~ 157.2°C. The molecular formula of **2** was deduced as C₁₆H₁₂O₇, through analysis of its HRESIMS spectrum, requiring eleven degrees of unsaturation. The UV spectrum of **2** showed absorptions at λ_{max} 382.8, 293.6, 264.4, and 237.2 nm. The IR spectrum of **2** showed absorption bands for OH group at 3403.2 cm⁻¹; C=O at 1640.6 cm⁻¹ and C=C aromatic at 1056.3 cm⁻¹. Investigation of 1D/2D NMR data of **2** (Table-2) revealed signals of two aromatic protons characteristic for 1,2,3,4-tetrasubstituted benzene, two aromatic singlets, one set of oxygenated methylene and one methoxy group, in addition to two carbonyl carbons and eight sp² hybridized carbons including four oxygenated ones. A comparison of its NMR data with those of published data for sydowinin B showed both compounds were identical¹⁸. Therefore, the structure of **2** was identified as sydowinin B (Fig.-2). This compound was initially found as a metabolite of *Aspergillus sydowii* more than thirty years ago¹⁹. Subsequent studies reported it from some species of marine-derived fungi, such as *A. sydowii* PSU-F154,²⁰ *Engyodontium album* DFFSCS021,²¹ *Aspergillus* sp. SCSIO Ind09F01,²² *Scopulariopsis* sp.²³ and, *Arthrinium* sp.,²³ as well as from fungal endophyte *Penicillium citrinum*.²⁵

Table-1: The ¹H and ¹³C NMR Data of Compound 1 in DMSO-*d*₆

No.	¹³ C (δ_c in ppm)	¹ H (δ_H in ppm, <i>J</i> in Hz)
1	170.2	-
2	38.7	3.71, d (15.6) 3.59, d (15,6)
3	135.2	-
4	110.9	6.17, d (2.3)
5	159.1	-
6	101.6	6.27, d (2.3)
7	157.3	-
8	119.6	-
9	206.0	-
10	42.9	2.98, ddd (15.6, 8.8, 2.5) 2.67, ddd (15.6, 9.8, 2.5)
11	22.3	1.63, m 1.45, m
12	26.2	1.33, m 1.18, m
13	23.3	1.35, m 1.20, m
14	31.6	1.37, m 1.54, m
15	71.4	4.83 dqd (9.1, 6.3, 2.7)
16	20.1	1.07, d (6.3)
7-OH	-	9.94, s
5-OH	-	9.75, s

Compounds **1** and **2** were subjected to biological assays for antibacterial activity against *S. aureus*, and MRSA (Table-3). They showed low antibacterial activity against *S. aureus* with MICs of 125 and 500 µg/disc and MRSA with MIC values of 250 and 500 µg/disc, respectively. Culvularin showed better antibacterial activity than sydowinin B. All isolated compounds were evaluated for their cytotoxicity against three cancer cell lines (Table-4). Culvularin had better cytotoxic activity against all cell lines including normal cell lines (Vero) than sydowinin B.

Based on literature studies, it is known that curvularin is a macrolide antibiotic produced by many fungal species from several genera, among which *Curvularia*, *Penicillium*, and *Alternaria*. This compound can inhibit the activity of protein 90 (HSP90). HSP90 is a chaperone that is involved in cell signaling, proliferation, and survival.²⁶ Therefore, curvularin is a promising compound in treating cancer, as well as potential antibiotics.²⁷

Table-2: Comparison of ^1H and ^{13}C NMR Data of Compound 2, with those reported for Sydowinin B ($\text{DMSO}-d_6$)

No.	^{13}C (δ_{C} in ppm)	^1H (δ_{H} in ppm, J in Hz)
1	117.2	-
2	148.9	-
3	125.4	7.47, d (9.1)
4	120.2	7.63, d (9.1)
4a	150.7	-
4b	155.5	-
5	103.9	6.99, d (1.3)
6	154.1	-
7	107.1	6.74, d (1.3)
8	160.4	-
8a	106.6	-
9	180.3	-
9a	117.0	-
10	166.6	-
11	52.2	3.85, s
12	62.4	4.59, d (6.0)
2-OH	-	10.40, s
8-OH	-	12.20, s
12-OH	-	5.52, t (6.0)

Fig.-2: Structures of Isolated Compounds from *P. oxalicum* WR3

Table-3: Antibacterial Activities of Curvularin and Sydowinin B from *P. oxalicum* WR3

The concentration of the compound ($\mu\text{g}/\text{disc}$)	Inhibition Zones (mm) $\pm$ Standard Deviation (SD)			
	<i>S. aureus</i>		MRSA	
	Curvularin	Sydowinin B	Curvularin	Sydowinin B
500	7.88 $\pm$ 0.04	7.88 $\pm$ 0.04	7.33 $\pm$ 0.04	7.28 $\pm$ 0.04
250	7.18 $\pm$ 0.04	-	7.23 $\pm$ 0.04	-
125	6.48 $\pm$ 0.11	-	-	-
62.5	-	-	-	-

Table-4: IC₅₀ Values of the isolated Compounds from *P. oxalicum* WR3 against Cancer Cell Lines and Vero Cell Line

Compound	T47D (µg/ml)	HeLa (µg/ml)	WiDR (µg/ml)	Vero (µg/ml)
Culvularin (1)	142.7	164.9	208.8	210.2
Sydowinin B (2)	454.5	> 100	557.8	> 100
Doxorubicin (positive control)	33.5	19.2	15.1	8.6

CONCLUSION

Culvularin and sydowinin B were isolated from the EtOAc extract of the marine sponge derived fungi, *P. oxalicum* (WR3). The compounds showed antibacterial activity with the MICs against *S. aureus* 125 and 500 µg/disc, respectively and the MIC values against Methicillin-Resistant *Staphylococcus aureus* 250 and 500 µg/disc, respectively. Culvularin showed higher cytotoxicity against T47D, HeLa, and WiDr cancer cell lines compared to sydowinin B.

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