SECONDARY METABOLITE FROM MANGROVE ENDOPHYTIC FUNGUS *Fusarium proliferatum* AED3

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ABSTRACT

As part of an ongoing search for secondary metabolites from an endophytic fungus *Fusarium proliferatum* AED3 derived from the mangrove plant *Ardisia elliptica*, a bioactive compound, was isolated by bioactivity-guided fractionation. The structure of the isolated compound was determined based on nuclear magnetic resonance spectroscopy and mass spectrometric data (UV, IR, LC-MS/MS, and NMR). The cytotoxic activity of the isolated compound was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against the T47D cancer cell line. Based on spectroscopic data, it was concluded that the isolated compound was beauvericin (1). This compound exhibited mild cytotoxic activity against the T47D cancer cell line with an IC₅₀ value of 112.2 μg/mL.

Keywords: Mangrove Plant, *Ardisia elliptica*, Cytotoxic Activity, T47D Cancer Cell Line, Beauvericin

INTRODUCTION

Many anti-cancer drugs are generally not selective against cancer cells. The drug could attack both cancer cells and normal cells. As a result, the administration of anticancer drugs is used to accompany several side effects, such as alopecia, gastrointestinal disease (nausea, vomiting, diarrhea), and bone marrow suppression.¹ Therefore, any efforts to discover new cancer agents are needed to resolve the problem. One of them is utilizing natural products derived from a unique ecosystem, such as mangroves. Mangroves have been widely used as a source of traditional medicine. Mangroves are also known as the host for various endophytic fungi. Endophytic fungi associated with mangroves are known to have the capability to produce secondary metabolites possessing a broad of pharmacological activities. Several researchers have reported that these microbes, which live within host organisms, are the trustworthy source of many bioactive compounds found in related marine invertebrates and mangroves.²

Some endophytic fungi from marine sponge and mangroves from West Sumatra, Indonesia, have been investigated by us, some of which were capable of producing compounds with promising antibacterial and cytotoxic activities.³-⁷ The anti-bacterial compound radicinin was found from endophytic fungus *Cochliobolus geniculatus* WR12. This compound can inhibit the growth of Methicillin-Resistant *Staphylococcus aureus* (MRSA) with a MIC value of 125 µg/disc. Radicinin exhibited moderate cytotoxic activity against Hela, T47D, and WiDr cell lines.⁸ Another example includes cytochalasin H from endophytic fungus *Diaporthe amygdali* SgKB4, which showed mild antibacterial activity against some pathogenic bacteria.⁹ Furthermore, chromatographic procedure on extracting an endophytic fungus *Penicillium oxalicum* WR3 resulted in the isolation of culvularin and sydowinin B, which revealed antibacterial activity against Staphylococcus aureus and MRSA with MIC values ranging from 125 to 500 µg/disc. Besides, both compounds showed cytotoxic activity, of which culvularin has higher cytotoxicity against the tested cell line than the activity of sydowinin B.¹⁰ In this study, we report cytotoxic activity and structure identification of bioactive metabolite derived from the endophytic fungus *Fusarium proliferatum* AED3. The fungus has been isolated from the mangrove plant *Ardisia elliptica*.¹¹
EXPERIMENTAL

Spectroscopic measurements used spectral grade solvents. TLC analysis was done on silica gel 60 F254 (Merck®) with detection under UV λ254nm and UV λ366nm wavelengths. The column chromatography was supplied by Silica gel 60 Merck®. The UV spectrum was measured on the Analytikjena® Ultraviolet-Visible S 210 Plus spectrometer. The infrared spectrum was recorded on the Perklin-Elmer Spectrum One FT-IR instrument. The mass spectrum was analyzed with the XEVO G2-S Q-TOF instrument equipped with Waters Acuity QSM. The NMR spectroscopy analysis was performed on Agilent 500 MHz with a DD2 console system that operates on a frequency of 500 MHz (1H) and 125 MHz (13C). T47D (human ductal breast epithelial tumor cell line) was obtained from the Laboratory of Biomedic, Faculty of Medicine, Andalas University, Padang, Indonesia.

Fungal Strain
The fungus F. proliferatum AED3 was isolated from the mangrove plant Ardisia elliptica. Fungus stocks were prepared on sabouraud dextrose agar (SDA) slants stored at 4°C.

Molecular Identification of The Endophytic Fungus
Fungal cultures were identified according to a molecular biological protocol by DNA amplification and sequencing using an internal transcribed spacer (ITS) DNA barcode with special primer pairs for fungi, following the procedures described before. The sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) program on National Center for Biotechnology Information (NCBI) database. MEGA 7.0 software is used to set up the phylogenetic trees.

Cultivation and Extraction of Secondary Metabolites
The fungus was grown by fermentation on a rice solid medium in a 1 L Erlenmeyer flask (to 100 g commercially available rice was added 110 ml of distilled water and kept overnight before autoclaving) at room temperature under static conditions. After 4 weeks of cultivation, the culture media were successively extracted with EtOAc. The EtOAc extract was evaporated under reduced pressure to give a crude extract (7.8 g). The extract was subjected to solvent–solvent partitioning using n-hexane and 90% MeOH, yielding a MeOH fraction that amounted to 3.1 g.

Isolation of Secondary Metabolite Compound
The MeOH fraction was separated by silica gel column chromatography using step gradient polarity with n-hexane 100%, n-hexane: EtOAc, EtOAc 100%, EtOAc: MeOH eluent to give eight subfractions (F1-F8). Subfraction F4 was further chromatographed over silica gel using an isocratic method and eluted with n-hexane: EtOAc (7:3) to obtain five subfractions (F4.1, F4.2, F4.3, F4.4, and F4.5). Subfraction F4.2 was recrystallized to afford compound 1 (10.5 mg). Moreover, subfraction F4.3 was applied to silica gel column chromatography to give three subfractions (F4.3.1, F4.3.2, and F4.3.3). Subfraction F4.3.2 was then recrystallized to afford compound 1 (4.1 mg).

MTT assay
Compound 1 was tested for its cytotoxic activity against the T47D cancer cell line by MTT assay. Cancer cells T47D were cultured in DMEM (GibcoTM) medium. The cells were seeded in a 96-well plate and incubated at 37°C in a CO2 incubator. After 24 h incubation (confluence), compound 1 was added to each well with the concentration of 100; 10; 1; and 0.1 µg/mL and incubated at 37°C in a CO2 incubator. After 24 h of action time, the medium was exchanged, and 10 µL of MTT reagent was added to each well and incubated for 4 h. The existence of formazan crystals was then dissolved in 50 µL DMSO and incubated for 30 min. The absorbance of compound 1 was measured using an ELISA reader at 540 nm. Culture medium without compound 1 was used as a negative control.

Statistical Analysis
Percentage of cell viability and value of Ln concentration were analyzed through regression equations for the calculation of IC50 value.
RESULTS AND DISCUSSION

Based on the BLAST search in the NCBI database, the AED3 isolate was identified as *Fusarium proliferatum* strain AED3. The phylogenetic tree was constructed by aligning and comparing all the 18S rRNA sequences with MEGA software version 7.0 (Fig-1).

![Fig-1: The Phylogenetic Tree of fungal isolate *Fusarium proliferatum* using Neighbor-joining method of the 18S rRNA Gene Sequence. The scale bar shows 0.0005 substitution Nucleotide Position](image)

Compound 1 was found as white gum. It exhibited a UV spectrum with a maximum wavelength at 205.60 nm (0.715). The IR spectrum displayed bands at the wavenumbers of 2972.07; 1756.65; 1659.92; and 1368.36 cm⁻¹, indicating the existence of C-H group, C=O (carbonyl ester), C=O (carbonyl amide).\(^{15,16}\)

The LC-MS/MS spectrum of compound 1 presented retention time at 13.81 min with precursor ion at m/z 784.41 [M+H]+, as showed in Fig-2. Precursor ion of 1 was fragmented to produce product ion at m/z 623.33 by separating fragment C\(_{10}\)H\(_{11}\)NO. Then, a was altered into b m/z 523.28 by removing fragment C\(_{5}\)H\(_{8}\)O\(_{2}\). Ion b was turned into c m/z 362.19 by separating fragment C\(_{10}\)H\(_{11}\)NO. Removal of fragment C\(_{5}\)H\(_{8}\)O\(_{2}\) from ion c gave the production d m/z 262.14. Finally, production e m/z 133.09 was formed by removing fragment C\(_{3}\)H\(_{6}\)O\(_{2}\) from d. The fragmentation model was presented in Fig-3. The fragmentation pattern of this compound was in line with that previously reported in the reference for beauvericin.\(^{17}\)

![Fig-2: The Chromatogram of Compound 1](image)

The NMR spectrum of compound 1 is exhibited by \(^1\)H NMR, \(^{13}\)C NMR, and DEPT. The DEPT spectra confirmed the presence of quarternary at \(\delta\)C 173.14; 138.04; 170.90 and methylene carbon at \(\delta\)C 35.40. The spectra data of 1 are shown in Table 1. Its spectra were compared to the literature and concluded that compound 1 was identical to beauvericin (Fig-4 and Table-1).\(^{18}\)
**ENDOPHYTIC FUNGUS** *Fusarium proliferatum*  

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**Fig.-3:** The Fragmentation Pattern of the Precursor Ion of Compound 1

**Fig.-4:** Chemical Structure of Compound 1

**Table-1:** The $^1$H and $^{13}$C NMR Data of Compound 1 compared to those reported in the Literature.

<table>
<thead>
<tr>
<th>No.</th>
<th>$^{13}$C of compound 1 in CD$_3$OD-d$_4$ (δ in ppm)</th>
<th>$^{13}$C of beauvericin in DMSO$^{18}$ (δ in ppm)</th>
<th>$^1$H of compound 1 in CD$_3$OD-d$_4$ (δ in ppm, $J$ in Hz)</th>
<th>$^1$H of beauvericin in DMSO (δ in ppm, $J$ in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>173.14</td>
<td>169.20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>57.84</td>
<td>55.69</td>
<td>5.72 (d, 8.5, 1H)</td>
<td>5.43 (dd, 12.0, 3.6, 1H)</td>
</tr>
<tr>
<td>2</td>
<td>35.40</td>
<td>33.99</td>
<td>3.07 (dd, 14.0, 4.0, 2H)</td>
<td>3.18 (dd, 14.4, 3.6, 2H)</td>
</tr>
<tr>
<td>3</td>
<td>138.04</td>
<td>136.51</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>129.68</td>
<td>128.51</td>
<td>7.18 (m, 1H)</td>
<td>7.22 (m, 1H)</td>
</tr>
<tr>
<td>5</td>
<td>129.83</td>
<td>128.17</td>
<td>7.18 (m, 1H)</td>
<td>7.24 (m, 1H)</td>
</tr>
<tr>
<td>6</td>
<td>127.98</td>
<td>126.44</td>
<td>7.10 (m, 1H)</td>
<td>7.17 (m, 1H)</td>
</tr>
<tr>
<td>7</td>
<td>129.83</td>
<td>128.17</td>
<td>7.18 (m, 1H)</td>
<td>7.24 (m, 1H)</td>
</tr>
<tr>
<td>8</td>
<td>129.68</td>
<td>128.51</td>
<td>7.18 (m, 1H)</td>
<td>7.22 (m, 1H)</td>
</tr>
<tr>
<td>9</td>
<td>32.18</td>
<td>31.32</td>
<td>3.23 (s, 3H)</td>
<td>3.02 (s, 3H)</td>
</tr>
</tbody>
</table>
Beauvericin was first isolated from the fungus *Beauveria bassiana*, while the fungus *Fusarium proliferatum* produced beauvericin was first isolated in 1994. Beauvericin is a cyclodepsipeptide compound that consists of three molecules of N-methyl phenylalanine (Phe) and three molecules of 2-hydroxyisovaleric acid (Hiv) in an alternating sequence.

Table-2: Citotoxicity of Compound 1 Against T47D Cancer Cell Line

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Absorption</th>
<th>Percent of Viability ± SD</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.352</td>
<td>41.0605 ± 1.87</td>
<td>112.2</td>
</tr>
<tr>
<td>10</td>
<td>0.687</td>
<td>91.2164 ± 1.96</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.884</td>
<td>120.7112 ± 0.70</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.938</td>
<td>128.7960 ± 2.09</td>
<td></td>
</tr>
</tbody>
</table>

The cytotoxic mechanism of beauvericin against those reported cancer cells involved the induction of ROS (Reactive Oxygen Species), which triggers an increase in oxidative stress and causes apoptotic cells. This compound also generates a signal of extracellular Ca<sup>2+</sup> into the cytosol, which raises intracellular Ca<sup>2+</sup> and produces cell death by apoptotic or necrotic pathway. The apoptotic pathway is also activated by a mitochondrial pathway in the signaling of MAPK, NF-κB, and p53. To date, beauvericin has been investigated against cancer cells. In our present study, the cytotoxic activity of beauvericin against the T47D cancer cell line was determined. The compound exhibited mild cytotoxic activity with an IC<sub>50</sub> of 112.2 µg/mL (Table 2). This is the first report on the cytotoxic activity of beauvericin towards the T47D cancer cell line.

**CONCLUSION**

Beauvericin was successfully isolated from the EtOAc extract of the mangrove plant-derived fungus *Fusarium proliferatum* AED3. The compound showed mild cytotoxic activity against T47D cancer cell lines. A study on the anticancer mechanism of action of beauvericin needs further investigation.

**ACKNOWLEDGEMENT**

We thank BOPTN of Andalas University, Padang, Indonesia for financial support, in the project of “Penelitian Dasar Unggulan Klaster Riset-Publikasi Guru Besar Universitas Andalas (PDU-KRP1GB-Uland)”, No. T/3/UN.16.17/PT.01.03/KOPDU-KRP1GB/2020.

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[RJC-6447/2021]