Antifungal Activity of (+)-2,2’-Epicytoskyrin A and Its Membrane-Disruptive Action

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Antifungal Activity of (+)-2,2′-Epicytoskyrin A and Its Membrane-Disruptive Action

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Abstract

(+)-2,2′-Epicytoskyrin A, a bis-anthraquinone isolated from fungal endophyte Diaporthe sp. GNB-10 associated with Uncaria gambir Roxb., was investigated for its antifungal activity. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) against 22 yeast strains and three filamentous fungi. The MICs of (+)-2,2′-epicytoskyrin A ranged from 16 to 128 µg/mL, which exhibited lower activity than the antifungal nystatin. A study of the mechanism of action revealed similar effects of (+)-2,2′-epicytoskyrin A and nystatin on Candida tropicalis at their MICs (16 and 8 µg/mL, respectively) and 2 times of the MIC. Both compounds caused cytoplasmic material and ion leakages on fungal cell, which were characterized by an increase in absorbance at 260 nm and 280 nm as well as Ca²⁺ and K⁺ ion concentrations. The morphology of the fungal cells after (+)-2,2′-epicytoskyrin A treatment was observed under a scanning electron microscope. The control cells, which were not treated with either (+)-2,2′-epicytoskyrin A or nystatin, showed a smooth surface, while the cells treated with either (+)-2,2′-epicytoskyrin A or nystatin shrank and displayed a donut-like shape. More shrinkage was observed in the 2 times MIC concentration and even more in the cells exposed to nystatin. The action of (+)-2,2′-epicytoskyrin A was proposed through membrane disruption.

Introduction

In recent years, plant-associated endophytes have received much attention due to their capability in producing various kinds of functional metabolites [1,2]. The discovery of taxol-producing endophytic fungi in 1993 by Stierle and his colleagues [3] gives a new perspective that plant-associated endophytes are able to produce similar metabolites as the host plant. This has driven many researchers to explore endophyte-colonizing medicinal plants that have been used by specific tribes for decades [4]. For example, researches have been...
conducted on endophytic fungi associated with medicinal plants of Kudremukh range in the Western Ghats of India [5], medicinal plants of Indonesia [6], and medicinal plants in traditional chinese medicine [7].

In our lab, research has been conducted on the screening of potential endophytes and their bioactive compounds for antimicrobials. During the work, we successfully isolated (++)-2,2’-epicytoskyrin A from endophytic fungus Diaporthe sp. GNBP-10 associated with the Gambier plant (Uncaria gambir Roxb.). The same compound was first isolated from Diaporthe sp. F associated with the tea plant of Camellia sinensis [8].

Research on the bioactivity of (++)-2,2’-epicytoskyrin A is scarce. Agusta et al., 2006 [8] reported moderate cytotoxic activity of (++)-2,2’-epicytoskyrin A against keratin-forming tumor cell line HeLa (KB) cells. Recently, we reported antibacterial activity of (++)-2,2’-epicytoskyrin A against 12 strains of pathogenic bacteria, including nine strains of clinical isolates of pathogenic bacteria isolated from humans with minimum inhibitory concentration (MIC) values ranging from 0.06 to 32 µg/mL [9]. In contrast, cytoskyrin A, an epimer of (++)-2,2’-epicytoskyrin A, demonstrated potent in vitro antibacterial activity against Gram-positive bacteria with MIC values ranging from 0.03 to 0.25 µg/mL [10]. Cytoskyrin A was also active against Candida albicans with an MIC of 16 µg/mL.

To the best of our knowledge, no study on antifungal activity has been reported for this compound. Here we report in vitro antifungal activity of (++)-2,2’-epicytoskyrin A. The mechanism of fungal growth inhibition was also studied.

**Methods**

(++)-2,2’-epicytoskyrin A. Diaporthe sp. culture used to produce (++)-2,2’-epicytoskyrin A was obtained after colony purification of endophytic fungi associated with Uncaria gambir Roxb. stems collected from Padang, West Sumatra as described in a previous paper [11]. The culture was deposited in the InaCC (the Indonesian Culture Collection) under the name GNBP-10 (InaCC No.208). The extraction, isolation, purification and identification of (++)-2,2’-epicytoskyrin A were described in our previous papers [9, 11].

**Fungi**. Twenty-two yeast and three filamentous fungi strains from 15 species were used in this study. The yeast strains were Candida albicans, C. catenulata, C. orthopsilosis, C. carpophila, C. rugosa, C. viswanathi, C. tropicalis, Pichia kudriavzevii, Hyphopichia burtonii, Rhodotorula minuta, Rhodosporidium toruloides, and Wickerhamomyces anomalus, and the filamentous fungi were Aspergillus niger, A. flavus and Fusarium oxysporum.

**Antifungal assay**. Antifungal activity of (++)-2,2’-epicytoskyrin A was determined with the broth microdilution method on 96-well plates [12]. The well was prepared by adding 100 µl of Sabouraud dextrose broth (SDB) (Difco, USA) and 100 µl of (++)-2,2’-epicytoskyrin A 512 µg/mL into the first row. Serial dilution was made in the subsequent rows so that the concentrations of (++)-2,2’-epicytoskyrin A ranged from 256 to 2 µg/mL. One hundred microliters of the fungal suspension at a concentration of 1 to 5 × 10^5 cfu/mL was added to each well. Nystatin (Sigma) 256 to 2 µg/mL were used as positive control. Growth control was prepared by replacing the (++)-2,2’-epicytoskyrin A solution with SDB medium, while negative control was prepared by replacing the (++)-2,2’-epicytoskyrin A solution with the solvent. The plate was then incubated at room temperature for 2 days followed by characterization using tetrazolium dye, methylthiazolyl-diphenyltetrazolium bromide (MTT) (Sigma, USA), colorimetric assay [13].

**Mechanism of action. Membrane disruption assay**. The 10 mL aliquot of C. tropicalis culture suspension in the SDB medium was centrifuged at 4 °C, 3,500 rpm for 20 min using the High Speed Refrigerated centrifuge Kubota 6500. The pelleted cell was re-suspended with 9 mL of phosphate buffer pH 7.0 (Sigma, USA) followed by the addition of 1 mL of either the solvent for the control test, (++)-2,2’-epicytoskyrin A solution at the final concentrations of 1 MIC and 2 MIC, or the nystatin solution at the final concentrations of 1 MIC and 2 MIC for positive control. After incubation in a shaker incubator at 25 °C for 48 h, the suspension was then centrifuged at 4 °C, 3500 rpm for 15 min. Supernatant fluid was analyzed for ion leakage as well as 260 and 280 nm absorbing materials, while the pellet cells were analyzed for morphology observation using a scanning electron microscope (SEM).

Analysis of 260 and 280 nm absorbing materials was carried out using a spectrophotometer (UV-1700 Pharmaspec 1700). Ion leakage (Ca2+ and K+ ion) analysis was measured with atomic absorption spectroscopy (AAS; Shimadzu AA-6800).

**Fungi cell morphology observation**. The cell pellets prepared as described above were consecutively soaked in 1% glutaraldehyde (Merck, Germany) for at least 4 h, 1% tannic acid in sodium cacodylate buffer (Wako, Japan) for at least 12 h, sodium cacodylate buffer two times for 10 min, and 1% osmium tetroxide (Merck, Germany) for 1 h. The pellets were then washed with 50% cold ethanol (Merck, Germany), left to stand for 10 min, and re-centrifuged. The pellets were washed consecutively with 50%, 70%, 80%, and 95% ethanol, ethanol absolute two times for 10 min, and tert-butanol (Merck, Germany) two times for 10 min. A drop of tert-butanol was added to precipitate the cells. The cells
were then smeared on a glass slip, coated with gold in vacuum condition, and observed under an electron microscope (JSM-5310LV).

**Assay of (+)-2,2'-epicytoskyrin A effect on filamentous fungi.** SDB medium was prepared with the addition of (+)-2,2'-epicytoskyrin A at ½ MIC and 1 MIC. The medium was then used to inoculate Aspergillus niger and A. flavus. The inoculum was incubated at room temperature for 7 days. On day 7, the fungi were prepared for SEM analysis using the same procedure as for C. tropicalis. Normal growth of fungi was also prepared with inoculation in SDB medium without the addition of (+)-2,2'-epicytoskyrin A.

**Results and Discussion**

(+)-2,2'-epicytoskyrin A showed low activity against several yeast strains and filamentous fungi on the antifungal microdilution broth assay. The MICs ranged from 16 to 128 µg/mL (Table 1). These MICs were higher than the MICs for commercial antifungal nystatin, except for the MIC against R. minuta (32 µg/mL). Similar activity was observed against filamentous fungi. The MICs of (+)-2,2'-epicytoskyrin A against A. niger and A. flavus were 4 times greater than those for nystatin, showing its lack of activity as an antifungal agent.

In terms of chemical structure, (+)-2,2'-epicytoskyrin A has a similar molecular structure as cytoskyrin A and rugulosin (Figure 1). (+)-2,2'-Epicytoskyrin A is an optical isomer of cytoskyrin A which differs only in the hydroxyl group position at C-2 and C-2'. Meanwhile, (+)-2,2'-epicytoskyrin A has a backbone similar to that of bis-anthaquinone with rugulosin but has methoxy instead of methyl as a substitution group. The antifungal activity of rugulosin has not been much explored. Brein et al., 1955, reported its activity in inhibiting the growth of the fungus Pythium intermedium. The results showed that rugulosin was able to prevent fungal growth on a Petri dish culture at concentrations up to 1:50 000 and gave partial inhibition as well as growth retardation at a concentration of 1:100 000 [14]. The activity of (+)-2,2'-epicytoskyrin A against C. albicans was low (MIC 48 µg/mL) compared to its epimer, cytoskyrin A, which had an MIC value of 16 µg/mL [10]. This indicates that hydroxy optical positioning might play a role in the activity.

Despite (+)-2,2'-epicytoskyrin A’s low activity, elucidating the mechanism of how the compound inhibits fungal growth is still interesting. In order to do that, ion and cytoplasmic material leakage as well as the morphology of the fungi, C. tropicalis, were determined after exposure to (+)-2,2'-epicytoskyrin A.

The release of cytoplasmic materials was established by absorbance measurement at 260 and 280 nm. The 260 nm absorbing material corresponds to DNA, while the 280 nm absorbing material corresponds to cellular protein. As the concentration of (+)-2,2'-epicytoskyrin A increased from 1 MIC (16 µg/mL) to 2 MIC, the absorbance of 260 and 280 nm absorbing material also increased (Figure 2). The same trend was also observed in the Ca²⁺ and K⁺ ion efflux (Figure 3). The ion concentration increased as the concentration of the compound increased. This was also observed in the treatment with the antifungal agent nystatin. In this experiment, however, it was observed that the concentration of Ca²⁺ ion in the fungal cells treated with nystatin 2 MIC was lower than that of (+)-2,2'-epicytoskyrin A. The possible explanation for this phenomenon is that the two compounds resulted in different severity of cell membrane damage. This might be correlated to the mechanism of action in more detail, which must be studied further. In comparison with the negative control, nystatin and (+)-2,2'-epicytoskyrin A showed higher concentrations of Ca²⁺ and K⁺ ions as well as cytoplasmic materials. This was in accordance with a previous study that demonstrated the release of cytoplasmic material from fungal cells treated with nystatin [15]. In the case of nystatin, this release occurred because nystatin bound to ergosterol in the fungi cell membrane, which led to disruption of the membrane permeability. The binding was believed to form barrel-like membrane-spanning ion channels [16].

The similar trend of cytoplasmic material leakage of (+)-2,2'-epicytoskyrin A and nystatin indicated that (+)-2,2'-epicytoskyrin A might also act by membrane disruption. However, whether (+)-2,2'-epicytoskyrin A binds to the fungal sterol as nystatin does or through other mechanisms, such as azole antibiotics, must be studied further. Azole compounds are also known to alter the sterol in the fungal cell membrane but through a different mechanism from nystatin. Sterols play an important role in fungal cell membrane integrity. The sterols need to be demethylated at C-4 in order to have this role, as in ergosterol [17]. The biosynthesis of ergosterol from lanosterol follows several steps starting with 14α-demethylation. Azole compounds act by impairing cytochrome P-450-dependent 14α-demethylase of lanosterol (a precursor of ergosterol) through either targeting heme protein, a co-catalysator in lanosterol demethylation, or directly inhibiting the enzyme [17]. As a result, fungal cells lack ergosterol, which is needed for the integrity of the cell membrane.

Another possible mechanism that causes disruption of the fungal cell membrane by (+)-2,2'-epicytoskyrin A is through oxidative stress. Julistiono et al., 2013, showed that the induction of oxidative stress could be one of the fungal toxicity mechanisms of (+)-2,2'-epicytoskyrin A. They showed that the addition of epigallocatechin gallate (EGCG) as an antioxidant can prevent the toxicity of epi-cytoskyrin A in Candida albicans [18]. Oxidative stress that was characterized by an increase in...
Table 1. Antifungal Activity of (+)-2,2’-Epicytoskyrin A

<table>
<thead>
<tr>
<th>Fungi</th>
<th>MIC (µg/mL) (+)-2,2’-epicytoskyrin A</th>
<th>Nystatin</th>
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<tbody>
<tr>
<td><em>Aspergillus flavus</em> InaCC F44</td>
<td>64</td>
<td>16</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> LIPIMC 759</td>
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<td>16</td>
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<tr>
<td><em>Candida albicans</em> LIPIMC Y0382</td>
<td>48</td>
<td>32</td>
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<tr>
<td><em>Candida carpophila</em> InaCC Y21</td>
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<td>16</td>
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<td><em>Candida carpophila</em> InaCC Y22</td>
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<td>8</td>
</tr>
<tr>
<td><em>Candida catenulata</em> InaCC Y4</td>
<td>128</td>
<td>16</td>
</tr>
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<td><em>Candida orthopsilosis</em> InaCC Y7</td>
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<tr>
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<td>8</td>
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<td><em>Candida orthopsilosis</em> InaCC Y11</td>
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<td>128</td>
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<td><em>Candida orthopsilosis</em> InaCC Y26</td>
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<td>128</td>
</tr>
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<td>8</td>
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<td><em>Candida rugosa</em> InaCC Y27</td>
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<td>16</td>
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<tr>
<td><em>Candida tropicalis</em> LIPIMC 0060</td>
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<td>8</td>
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<tr>
<td><em>Candida viswanathii</em> InaCC Y36</td>
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<td><em>Hyphochiia burtonii</em> InaCC Y39</td>
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<td>8</td>
</tr>
<tr>
<td><em>Pichia kudriavzevii</em> InaCC Y23</td>
<td>32</td>
<td>4</td>
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<tr>
<td><em>Wickerhamomyces anomalous</em> InaCC Y37</td>
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<table>
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<th>Filamentous fungi</th>
<th>MIC (µg/mL)</th>
<th>Nystatin</th>
</tr>
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<td><em>Fusarium oxysporum</em> InaCC F78</td>
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<td><em>Rhodosporidium toruloides</em> InaCC Y57</td>
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<td>64</td>
</tr>
<tr>
<td><em>Rhodotorula minuta</em> IAM 1296</td>
<td>32</td>
<td>64</td>
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</tbody>
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Figure 1. Chemical Structures of (+)-2,2’-epicytoskyrin A, Cytoskyrin A and Rugulosin

reactive oxygen species (ROS) could be one of the possible mechanisms of cell death [19]. The increase in ROS in a cell can cause an increase in membrane fluidity. ROS react freely with unsaturated lipids that constitute most of the fungal membrane, resulting in polar lipid hydroperoxides that disturb the hydrophobic phospholipid membrane [20]. Morphological observation under SEM revealed the shrinkage of the cells that appeared as a donut-like shape (Figure 4). The shrinkage was observed more in the concentration of 2 MIC and even more in the cells exposed to nystatin. This could occur because the release of cytoplasmic materials was more rapid than the entrance of the osmotic stabilizer [21]. In the case of
Figure 2. Effect of (+)-2,2'-epicytoskyrin A on the Membrane Integrity of *C. tropicalis* LIPIMC 60 Indicated by the Release of Absorbing Material at 260 nm (A) and 280 nm (B)

Figure 3. Effect of (+)-2,2'-epicytoskyrin A on the Membrane Integrity of *C. tropicalis* LIPIMC 60 Indicated by the Release of Ca\(^{2+}\) and K\(^+\) Ions

Figure 4. Effect of (+)-2,2'-epicytoskyrin A on the Cellular Morphology of *C. tropicalis* LIPIMC 60. A. Control, B. (+)-2,2'-Epicytoskyrin A 1 MIC, C. (+)-2,2'-Epicytoskyrin A 2 MIC, D. Nystatin 1 MIC, The Treatment resulted in Cell Shrinkage that Appeared as a donut-like Shape (indicated by the White Arrow). The Magnification of All Images is 2000X
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Figure 5. Effect of (+)-2,2’-epicytoskyrin A on Filamentous Fungi Aspergillus flavus and A. niger. A. Aspergillus flavus Inoculum in SDB Medium as a control (left), (+)-2,2’-epicytoskyrin A ½ MIC (32 µg/mL) (middle), and (+)-2,2’-epicytoskyrin A 1 MIC (64 µg/mL) (right); B. Aspergillus niger in Control SDB Medium under SEM; C. Aspergillus niger in (+)-2,2’-epicytoskyrin A ½ MIC under SEM, 1500X Magnification

nystatin, however, shrinkage can be followed by swelling and bursting of the cells in high concentrations of nystatin up to 20 µg/mL [22].

In a study on the effect of (+)-2,2’-epicytoskyrin A on filamentous fungi showed no growth at the MIC, while normal fungal growth was observed on the negative control (Figure 5, A). The culture treated with ½ MIC of (+)-2,2’-epicytoskyrin A also showed fungal growth, but it was slowed. The culture exhibited spore germination inhibition due to the compound. At the microscopic level, the effect of (+)-2,2’-epicytoskyrin A was hardly seen. The control and the treated fungi showed similar morphology. Based on this experiment, (+)-2,2’-epicytoskyrin A can inhibit spore germination in filamentous fungi.

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