In Vitro Antifungal Activity of Naphthoquinone Derivatives

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Fungal infection in human can be classified as superficial and systemic mycoses, depending on which areas of the body are primarily affected. Systemic fungal infections are increasingly serious causes of high morbidity and mortality of hospitalized patients with impaired immune systems brought about by the use of cytotoxic drugs, immunosuppressive therapy, or human immunodeficiency virus infection. However azole derivatives including fluconazole and itraconazole are widely used in clinical settings but there are major weaknesses in their spectra, potency, safety and pharmacokinetic properties. In addition, the emergence of fungal strains resistant to existing antifungal drugs, especially fluconazole which is most commonly used, is becoming a significant problem. Thus the development of new, effective antifungal agents is strongly needed in medicine. The possibility of antifungal drugs for use in the prevention and treatment against oral candidosis in human immunodeficiency virus-positive patients or terminally ill patients has also been reported. Shikon is widely used as a material to prepare an ointment called “Shiun-ko” which is used to treat wounds, burns and hemorrhoids in Japan. We previously reported that the extracts containing the pigments of Lithospermum erythrorhizon (Ko-Shikon) and Arnebia euchroma (Nan-Shikon), which are available in the Japanesemarket using “Shikon” showed antifungal activities against Candida albicans ATCC24433; acetylsikokinon also inhibited fungal growth at the minimal inhibitory concentration (MIC) of 15.6 μg/ml (RPMI24h) or 3.9 μg/ml (YNB24h). Although many pharmacological studies of shikon and its constituents have been published, our report was the first evidence about antifungal activity against fungal pathogens. The present study was carried out to develop the antifungal properties of naphthoquinone derivatives constituting Lithospermum erythrorhizon against several fungal pathogens.

Materials and Methods

Preparation of the Extracts and Tested Compounds Lithospermum erythrorhizon marketed as shikon was purchased from Tochimoto-Tenkai-do (Osaka). Deoxyshikonin, acetylsikkain, β-hydroxyisovaleryl shikonin and shikonin were isolated from the chloroform extracts of L. erythrorhizon as described previously. Fluconazole (0.2% Diflucan inj.), purchased from Pfizer Co., Ltd. was used as a standard agent in this study. Concentrations were adjusted for 6.4 mg/ml of each compound with 100% dimethyl sulfoxide and diluted with RPMI 1640 broth (Sigma Chemical, St. Louis, MO, U.S.A.) for 50-fold final strength. For antifungal susceptive test, a two-fold dilution series of each compound was prepared at 100-fold final strength.

Microorganisms Candida albicans YFC 497, YFC 803 (azole resistant), C. glabrata YFC 501, C. krusei YFC 827, C. tropicalis YFC 052, C. parapsilosis YFC 826, Saccharomyces cerevisiae YFC 250, Cryptococcus neoformans YFC 513, Trichosporon cutaneum YFC 517, and Aspergillus fumigatus YFC 526 were used for the determination of the antifungal spectrum. All of the strains are maintained at −80 °C in RPMI 1640 (pH 7.1).

Assay of MIC 1) Except for Aspergillus fumigatus, MIC values were determined by serial diluting methods in liquid media using a microtiter plate following the method of the National Committee for Clinical Laboratory Standards (NCCLS). MIC was defined as the lowest concentration of a test substance causing no detectable growth of a test microorganism compared with the blank experiment (optical density (OD) at 595 nm) after the present incubation time. MICs were evaluated after incubation for 48 h at 35 °C (except for Cryptococcus neoformans which was for 72 h at 35 °C) and were adjusted to 2×10⁴ cells/ml.

2) For A. fumigatus, MIC values were determined by serial diluting methods in liquid media using a microtiter plate following the method of the NCCLS. Other steps were the same as described above but cells were adjusted to 2×10⁴ cells/ml.
RESULTS AND DISCUSSION

Antifungal activities against fungal pathogens of naphthoquinone derivatives were investigated by measuring their MIC using the NCCLS macrobroth dilution method. Fluconazole was used as a standard for the evaluation of their activities. As indicated in Table 1, each naphthoquinone exhibited several degrees of antifungal activity. Shikonin was found to have a four-fold stronger fungicidal activity (MIC of 4 µg/ml) than fluconazole against yeast-like fungi Candida krusei and two-fold stronger (MIC of 4 µg/ml) againstSaccharomyces cerevisiae though it showed the same potency against C. glabrata. Deoxyshikonin also exhibited four-fold stronger activity against C. krusei (MIC of 4 µg/ml) and three-fold (MIC of 2 µg/ml) against Saccharomyces cerevisiae. Acetylshikonin and β-hydroxyisovalerylshikonin showed lower activities against all fungal pathogens except for C. krusei compared with the standard, but still exhibited some antifungal activity. Against the filamentous fungus, Trichosporon cutaneum, all naphthoquinones were found to have a range of activities with lower potency than standard. When deoxyshikonin and β-hydroxyisovalerylshikonin were applied to this assay against azole-resistant C. albicans YFC 830, these activities were the same as that of fluconazole. These results suggest that shikonin and deoxyshikonin are notable compounds and/or important candidates of lead compounds for new anti-fungal agents, because they are quite active despite their contents being considerably lower than acetylshikonin. Previous we reported that acetylshikonin showed antifungal activity against Candida albicans ATCC24433 at MIC: 15.6 µg/ml (RPMI_1640) and MIC: 3.9 µg/ml (YNB_200) suggesting the antifungal activity of shikon was concentrated in constituting naphthoquinone-pigments. This study demonstrated details of antifungal spectra to the fungal pathogens. Shikonin and its derivatives had been reported to have various pharmacological effects: an anti-inflammatory effect, enhancement of the proliferation of granular tissue, anticancer promotion and anti-bacterial activity. Its anti-inflammatory effects were examined in detail and found to have useful medical applications. However, when the biological activity of these compounds was tested against soil-borne bacteria and fungi, a wide range of sensitivities was observed but there was no evidence of their activity against fungal pathogens. We found a wide range of activity against fungal pathogens in this study. This activity seems potentially useful for clinical applications on inflammatory agents with antibacterial and antifungal activity: it could be applicable for caries control and oral candidosis following the use of cytotoxic drugs, immunosuppressive therapy, and human immunodeficiency virus infection, or fungal infection in a compromised host with an opportunistic infection which does not allow use of steroidal anti-inflammatory drugs. Also, it was reported recently that Candida albicans is present at the occurrence of periodontal pathogenic bacteria, suggesting that antifungal agents would prevent periodontal pathogens. Specifically, Shikon would not only be a significant candidate for treatment of inflammation but also elsewhere in the field of periodontology. The importance of naphthoquinone-derivatives constituting Shikon on antifungal activity at an in vivo level will be elucidated in a future study, and may suggest they could be a useful medicine for inflammatory agents which have antifungal activity.

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REFERENCES AND NOTES

1) Present address: Do · Planning. Ltd., 70–5 Nakayashiki, Kadonowaki, Ishinomaki, Miyagi 986–0653, Japan.

Table 1. In Vitro Antifungal Activities of Naphthoquinone-Derivatives against Fungal Pathogens

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (µg/ml)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>FLCZ</th>
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<tbody>
<tr>
<td>Candida albicans</td>
<td>YFC 497</td>
<td>&gt;64</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>0.13</td>
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<tr>
<td>C. albicans</td>
<td>YFC 830</td>
<td>&gt;64</td>
<td>32</td>
<td>32</td>
<td>&gt;64</td>
<td>32</td>
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<tr>
<td>C. glabrata</td>
<td>YFC 501</td>
<td>16</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>C. krusei</td>
<td>YFC 827</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>YFC 052</td>
<td>32</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>YFC 826</td>
<td>32</td>
<td>16</td>
<td>32</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>YFC 250</td>
<td>2</td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>YFC 513</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Trichosporon cutaneum</td>
<td>YFC 517</td>
<td>8</td>
<td>16</td>
<td>32</td>
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<td>4</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>YFC 526</td>
<td>&gt;64</td>
<td>32</td>
<td>64</td>
<td>&gt;64</td>
<td>64</td>
</tr>
</tbody>
</table>

1. deoxyshikonin; 2. acetylshikonin; 3. β-hydroxyisovalerylshikonin; 4. shikonin; FLCZ, fluconazole.