Anti-Aspergillus Activities of the Ligusticum chuanxiong Essential Oil Alone and in Combination with Antibiotics

Youn Sim and Seungwon Shin*

College of Pharmacy, Duksung Women’s University, Seoul 132-714, Korea

Abstract – The present study aimed to assess the antifungal properties of the essential oil fraction from Ligusticum chuanxiong (Umbelliferae) and its components against five clinically important Aspergillus species. The essential oil fraction was extracted from the underground parts of the plant by steam distillation, and its main components, namely, Z-ligustilide, butylidene phthalide, and p-cresol were isolated by column chromatography. The antifungal activities of the essential oils were evaluated by the broth dilution method. Both the total essential oil fraction of L. chuanxiong and its components showed significant anti-Aspergillus activity against all five tested strains with MICs between 62.5 and 250 µg/ml, respectively. In a checkerboard microtiter assay, the combination of antibiotics, itraconazole with the essential oil fraction of L. chuanxiong or its main components exhibited synergistic or additive, and in some cases indifferent, effects against the tested Aspergillus species, resulting in FICIs (fractional inhibiting concentration indices) ranging from 0.12 to 2, while the combination of antibiotics, amphotericin B with L. chuanxiong essential oils mostly showed antagonistic effects.

Keywords – Aspergillus spp., Ligusticum chuanxiong, essential oils, Z-ligustilide, butylidene phthalide, itraconazole, p-cresol, amphotericin B, synergism

Introduction

Ligusticum chuanxiong Hort. (Umbelliferae), a perennial herb cultivated mainly in Korea and China, is one of the main plant sources of Cnidii Rhizoma, which has been used in traditional medicine for the treatment of headaches, abdominal pain, and menstrual disorders (Packer et al., 2004). The essential oil of this herb contains certain compounds such as phthalides, ligustilide, butylidene phthalide, cnidilide, and others that were shown to exhibit cardiovascular, antiplatelet, anti-inflammatory, and also antimicrobial and insecticidal effects (Beck and Chou, 2007; Zhang et al., 2007, Sim and Shin, 2008; Wang et al., 2010).

Although many higher plants produce antifungal compounds, few plant-derived agents have been evaluated for their activity against human pathogenic fungi. The development of natural antifungal agents is a very attractive prospect, in particular because the currently available therapeutic agents against mycoses have several drawbacks including toxicity, rapid development of resistance and drug-drug interactions (Hachem et al., 2004; Cuenca-Estrella et al., 2005; Beernaert et al., 2009; Vanhee et al., 2010; Xu et al., 2010). Essential oils are one of the most promising groups of natural compounds for the development of new antifungal agents despite their malabsorption from the human intestine and relatively mild activities compared to synthetic antifungal drugs, which may ultimately limit their clinical application in systemic fungal infections (Bidlack et al., 2000; Shin and Lim, 2004).

Aspergillus species cause a number of severe diseases, both in the normal and the immunocompromised host, encompassed under the name aspergillosis and including allergic disease, saprophytic disease, superficial infections and invasive infections (Denning, 1996; Shin, 2003; Yuchong et al., 2010; Winterstein et al., 2010). Among Aspergillus species, A. fumigatus is the most common human infectious agent, followed by A. flavus, A. niger and A. terreus (Xavier et al., 2008; Dagenais et al. 2009). A. versicolor produces many toxic compounds, which can cause severe symptoms in humans and animals infected through inhalation or other forms of contact with debris or spores (Engelhart, et al., 2002; Veraldi et al., 2010). Interestingly, a recent report describes the isolation of bioactive compounds from A. versicolor that show promise for the development of anticancer drugs (Lee et al., 2010).
The polyene antifungal amphotericin B is the drug of choice for the treatment of aspergillosis (Scholar and Pratt, 2000). However, this agent is toxic in its conventional form and very expensive in its lipidic form (Otsubo et al., 1999; Ibrahim et al., 2010; Lestner et al., 2010). Itraconazole, which is an alternative antifungal treatment against various Aspergillus infections, is better tolerated than other antifungal agents and more active than other azoles, although it has shown certain mild and transient adverse effects mostly affecting the liver (Srebrnik et al., 2005).

In the present study, the antifungal activities of the essential oil of L. chuanxiong and its main components, Z-ligustilide and butylidene phthalide were evaluated against five important pathogenic Aspergillus species by broth dilution tests. The potentially synergistic effects of essential oils and synthetic antifungal drugs were assessed by combining essential oils with amphotericin B or itraconazole.

**Experimental**

**Sample preparation for testing antifungal activities and fungal strains** — Butylidene phthalide, Z-ligustilide, and p-cresol were isolated from the essential oil fraction, which was extracted from the dried underground parts of L. chuanxiong by steam distillation (Sim and Shin, 2008). Itraconazole and amphotericin B were purchased from Sigma Chemical Co., USA. Fungal organisms were obtained from the Korean Culture Center of Microorganisms (KCCM). A. flavus KCCM 11899, A. fumigatus KCCM 60027, A. niger KCCM 11241, A. terreus KCCM 12067, and A. versicolor KCCM 11592 were cultured in yeast and malt extract broth (YM) or malt extract liquid medium for six or seven days at 26 °C. The turbidity of the cell suspension was measured at 600 nm and adjusted with medium to match that of a 0.5 McFarland standard (10²–10⁶ colony forming units (CFU)/ml).

![Butylidene phthalide](image1)

Butylidene phthalide

![Z-Ligustilide](image2)

Z-Ligustilide

**Determination of minimal inhibitory concentration (MIC)** — Essential oil samples were serially diluted with 10% (v/v) dimethyl sulfoxide (DMSO) to obtain solutions that contained from 0.39 to 50 mg/ml essential oil, to which 10 µl Tween 80 was added. After shaking, 5-µl aliquots of the essential oil solutions were added to the wells of 96-well microtiter plates. A 100-µl suspension of A. niger or A. flavus, adjusted to 10⁶–10⁷ CFU, was then added to individual wells and cultivated at 26 °C. The MIC was defined as the lowest concentration that completely inhibited visible fungal growth after 6–7 days. Each organism was also cultured with a blank solution containing Tween 80 and DMSO, at concentrations equivalent to those in the test solutions, to certify that these vehicles did not affect fungal growth. Values shown are the means of tests performed in triplicate.

**Checkerboard titration test** — Ten serial two-fold dilutions of essential oil or antibiotics were prepared using the same solvents as those used in the MIC tests. The 5 µl - aliquots of each L. chuanxiong oil dilution were added to the wells of a 96-well plate in a vertical orientation and 5-µl aliquots of each amphotericin B or itraconazole dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the two compounds. Each well was then inoculated with 100 µl (ca. 5 × 10⁴ CFU/well) of one of the two Aspergillus fungal suspensions and cultivated at 26 °C. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of each essential oil and itraconazole, divided by the MIC of oil or itraconazole alone. The FIC index (FICI), obtained by adding both FICs, was interpreted as representing a synergistic effect when it was ≤ 0.5, as additive or indifferent when it was > 0.5 and ≤ 2.0, and as antagonistic when it was > 2.0 (Davidson et al., 1989). Similar checkerboard experiments were also performed using amphotericin B.

**Results and Discussion**

The inhalation of Aspergillus conidia from the environment can cause different forms of aspergillosis including allergic bronchopulmonary aspergillosis, pulmonary aspergillosis, or invasive aspergillosis. Invasive aspergillosis is an acute and severe disease that predominantly occurs in immunocompromised individuals, those with weakened immune systems, or patients who have received large amounts of antibiotics (Zhirong et al., 1999; Lin et al., 2006). Plant essential oils are an attractive source for the development of new anti-Aspergillus drugs. They are potential to easily diffuse into the atmosphere and respiratory system.

In the present study, the antifungal activity of the essential oil fraction of L. chuanxiong against five clinically important Aspergillus species was evaluated and the five
components of the oil were compared to provide a rationale for the development of new natural drugs for the treatment and prophylaxis of aspergillosis.

In a previous report, \( Z \)-ligustilide was identified as the predominant component of the essential oil fraction of \( L. \) chuanxiong, comprising over 40% of this oil (Sim and Shin, 2008). Butylidene phthalide and \( p \)-cresol were the next most abundant compounds. These three compounds were isolated by column chromatography and used in this study. The MICs of the essential oils and the two antifungal drugs commonly used for the treatment of aspergillosis due to their strong capacity to inhibit the growth of the \( Aspergillus \) species, amphotericin B and itraconazole, are listed in Table 1.

The total oil fraction and main components of \( L. \) chuanxiong, showed significant inhibitory activity against five species of \( Aspergillus \). MICs ranged from 62.5 to 125 \( \mu g/ml \), except in tests against \( A. \) flavus. MICs determined for antibiotics, amphotericin B and itraconazole were 4–32 \( \mu g/ml \) and 0.25–4 \( \mu g/ml \), respectively. Among the \( Aspergillus \) species tested, \( A. \) terrus showed the highest sensitivity to itraconazole, exhibiting a MIC of 0.25 \( \mu g/ml \). However, this species had the highest MIC (32 \( \mu g/ml \)) for amphotericin B. The differences in susceptibility to the drugs among the species could be related to differences in the composition of the cell membrane of the fungi. In all tests, the two antibiotics showed significantly higher activity than the essential oils.

To assess whether combination treatment with the most active main components of the oil, \( Z \)-ligustilide, butylidene phthalide, or \( L. \) chuanxiong essential oil fraction with antibiotics could enhance the antifungal activity and facilitate the use of lower concentrations of antibiotics, checkerboard titers were performed combining the \( L. \) chuanxiong essential oils with amphotericin B or itraconazole.

As listed in Table 2, FICIs against \( Aspergillus \) species ranged between 0.12 and 2 for itraconazole combined with \( Z \)-ligustilide or butylidene phthalide, indicating synergistic, additive, or indifferent effects of the antibiotic

<table>
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<tr>
<th>Essential Oils</th>
<th>MIC (( \mu g/ml ))</th>
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<tbody>
<tr>
<td></td>
<td>( A. ) flavus</td>
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<tr>
<td>Essential oil fraction</td>
<td>250</td>
</tr>
<tr>
<td>( Z )-Ligustilide</td>
<td>125</td>
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<tr>
<td>Butylidene phthalide</td>
<td>125</td>
</tr>
<tr>
<td>( p )-cresol</td>
<td>250</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>4</td>
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<tr>
<td>Itraconazole</td>
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<tr>
<th>ESSENTIAL OILS</th>
<th>MIC (( \mu g/ml ))</th>
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<tr>
<td></td>
<td>( A. ) flavus</td>
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<tr>
<td>Essential oil fraction</td>
<td>0.50</td>
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<tr>
<td>Itraconazole</td>
<td>0.25</td>
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<tr>
<td>( Z )-Ligustilide</td>
<td>1</td>
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<tr>
<td>Itraconazole</td>
<td>1</td>
</tr>
<tr>
<td>Butylidene phthalide</td>
<td>0.5</td>
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<tr>
<td>Itraconazole</td>
<td>0.25</td>
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FIC (Fractional inhibitory concentration) = MIC tested in combination / MIC tested with single sample alone.
FICI = FIC of \( L. \) chuanxiong essential oil or oil component + FIC of itraconazole.
and the essential oil compounds. Among the three tested oils, butylidene phthalide showed the most distinct synergism when combined with itraconazole, resulting in FICIs between 0.12 and 0.325 against *A. niger*, *A. fumigatus*, *A. terreus*, and *A. versicolor*. Isobolograms were constructed using MIC data derived from the combination of itraconazole and *Z*-ligustilide in various concentration combinations. As shown in Fig. 1, isobolograms for *A. fumigatus* (A) and *A. niger* (B) based on their FICI values in checkerboard titer tests of 0.31 and 0.12, respectively, showed a curve distinctly deviated to the left, confirming the presence of synergistic anti-fungal activity (Davidson and Parish, 1989). In similar tests with amphotericin B, the two agents used showed an antagonistic relationship. FICIs could not be calculated because the MICs exceeded the tested concentrations of amphotericin B. The addition of oils to the fungal cultures containing this antibiotic were inhibited the antifungal effects of amphotericin B, whereas the antibiotic did not affect the activity of the *Ligusticum* essential oil. The mechanism underlying this antagonistic effect could not be explained; however, the possibility that the oil could prevent the amphotericin B from reaching the fungal cell membrane is being considered.

In conclusion, the present data indicate that components of the essential oil fraction from *L. chuanxiong* may be useful agents for the treatment and prophylaxis against aspergillosis. In addition, the improvement in anti-*Aspergillus* activity by itraconazole administered in combination with *Z*-ligustilide or butylidene phthalide could provide alternative therapies to enhance the efficacy of itraconazole in the treatment of aspergillosis. Further studies will be required to evaluate the value of these essential oils for the development of potential therapies.

**Acknowledgements**

This study was supported by a grant from Duksung Women's University (2009). The author acknowledges the support.

**References**


