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

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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, amphothericin B with *L. chuanxiong* essential oils mostly showed antagonistic effects.
Keywords – *Aspergillus* spp., *Ligusticum chuanxiong*, essential oils, *Z*-ligustilide, butylidene phthalide, itraconazole, *p*-cresol, amphothericin 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).

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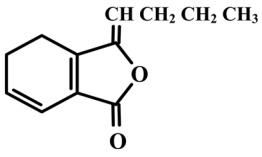
The polyene antifungal amphotericin B is the drug of choice for the treatment of aspergillosis (Scholar and Pratt, 2000). However, this agent is toxic in its conventional form and very expensive in its lipidic form (Otsubo *et al*., 1999; Ibrahim *et al*., 2010; Lestner *et al*., 2010). Itraconazole, 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).

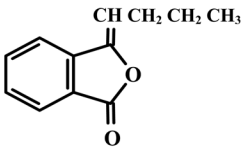
In the present study, the antifungal activities of the essential oil of *L. chuanxiong* and its main components, *Z*-ligustilide and butylidene phthalide were evaluated against five important pathogenic *Aspergillus* species by broth dilution tests. The potentially synergistic effects of essential oils 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 (10^5~10^6 colony forming units (CFU)/ml).



Butylidene phthalide



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 10^4~10^5 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 titer 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. 5×10^4 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 aspergilloma, 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

Table 1. MICs of *L. chuanxiong* essential oils against *Aspergillus spp*

Essential Oils	MIC (ug/ml)				
	A. flavus	*A. fumigatus*	*A. niger*	*A. terreus*	*A. versicolor*
Essential oil fraction	250	62.5	125	125	125
Z-Ligustilide	125	62.5	125	125	125
Butylidene phthalide	125	62.5	125	62.5	62.5
p-cresol	250	250	250	250	125
Amphotericin B	4	8	8	32	4
Itraconazole	2	2	4	0.25	1

Table 2. Fractional inhibitory concentrations (FICs) and FIC indices (FICIs) of *L. chuanxiong* essential oil in combination with itraconazole against *Aspergillus* spp

Sample	*A. flavus*		*A. fumigaus*		*A. niger*		*A. terreus*		*A. versicolor*	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
Essential oil fraction	0.50	0.75	0.50	1.50	0.50	0.75	0.50	1	1	2
Itraconazole	0.25		1		0.25		0.50		1	
Z-Ligustilide	1	2	0.50	1.50	0.50	0.625	0.50	0.75	0.50	1
Itraconazole	1		1		0.125		0.25		0.50	
Butylidene phthalide	0.5	0.75	0.25	0.31	0.06	0.12	0.125	0.325	0.25	0.325
Itraconazole	0.25		0.06		0.06		0.25		0.125	

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.

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 in value from 62.5~250 μg/ml, indicating differences in susceptibility among the species tested. In similarity with the results of the study using *Trichophyton* previously reported by Sim and Shin (2008), the MICs of the total oil fraction were similar or higher than the MICs of *Z*-ligustilide, indicating that the activity of the oil fraction is due largely to *Z*-ligustilide. Butylidene phthalide showed a higher activity than *Z*-ligustilide, the total oil fraction, and *p*-cresol, with MICs ranging from 62.5 to 125 μg/ml, except in tests against *A. fumigatus.* MICs determined for antibiotics, amphotericin B and itraconazole were 4~32 μg/ml and 0.25~4 μg/ml, respectively. Among the *Aspergillus* species tested, *A. terrus* showed the highest sensitivity to itraconazole, exhibiting a MIC of 0.25 μg/ml. However, this species had the highest MIC (32 μg/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, thus minimizing the potential side effects of these drugs, checkerboard titer tests 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

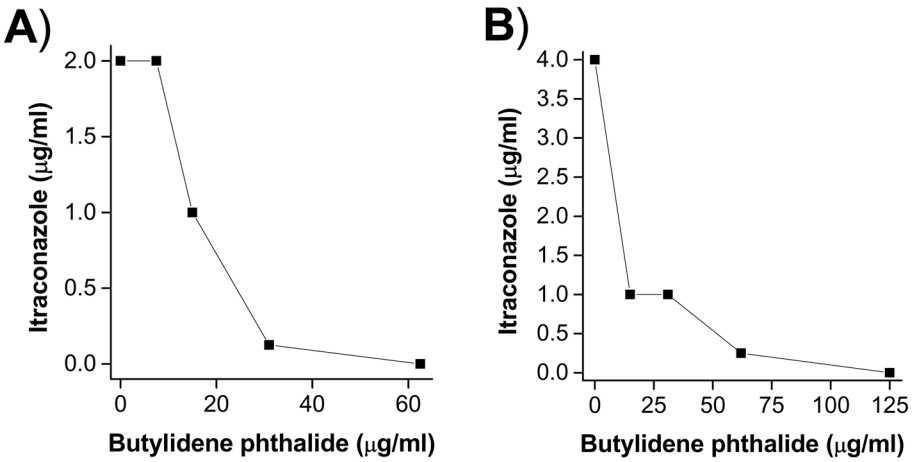


Fig. 1. Isobolograms of butylidene phthalide combined with itraconazole against *A. flumigatus* (A) and *A. niger* (B).

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 amphothericin 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 butilidene 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.

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