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Anti-*Aspergillus* Activities of Plant Essential Oils and Their Combination Effects with Ketoconazole or Amphotericin B

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The essential oils from *Cedrus atlantica, Styrax tonkinensis, Juniperus communis, Lavandula angustifolia, Melaleuca alternifolia, Pelargonium graveolens, Pogestemon patchouli* and *Rosmarinus officinalis* were analyzed by GC-MS. Antifungal activities of the oils were investigated by disk diffusion assay and the broth dilution method against *Aspergillus niger* and *A. flavus*. The effects of geraniol and the essential oil fraction from *P. graveolens* on the antifungal activity of amphotericin B and ketoconazole were examined using a checkerboard microtiter assay against both *Aspergillus* fungi. Most of the tested essential oils, with the exception of *C. atlantica, J. communis,* and *P. patchouli,* significantly inhibited growth of *A. niger* and to a lesser extent that of *A. flavus,* with MICs (minimal inhibitory concentrations) in the range 0.78-12.5 mg/mL. The essential oil fraction of *P. graveolens* and its main components, geraniol and citronellol, exhibited additive effects with amphotericin B and with ketoconazole against both *Aspergillus* species, resulting in fractional inhibitory concentration (FIC) indices ranging from 0.52 to 1.00.

Key words: *Aspergillus niger, A. flavus,* Essential oils, Amphotericin B, Ketoconazole, Geraniol, Citronellol, Synergism

INTRODUCTION

Antifungal agents appear to be prevalent among the higher plants, but very 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 since currently available therapeutics against mycoses have several drawbacks including toxicity, rapid development of resistance and drug-drug interactions (Gundidza, 1993; Hammer *et al.*, 1998; Shahi *et al.*, 1999; Giordani *et al.*, 2001). Essential oils have been one of the most promising groups of natural compounds from which a new prototype of antifungal agents may be developed (Yoon *et al.*, 1994). This is true, despite the fact that their malabsorption from the human intestine and relatively mild activities compared to commercial, synthetic antifungal drugs may ultimately limit their clinical application in systemic fungal infection (Garg and Siddiqui, 1998; Adam *et al.*, 1999; Bidlack *et al.*, 2000; Shin and Kang, 2003).

In this study, the essential oils which have been recommended for the treatment of fungal infections in aromatherapy and complementary medicine, were analyzed and compared by gas chromatography-mass spectrometry (GC-MS). On the basis of these results, the antifungal activities of the essential oils and their main components were evaluated against *Aspergillus niger* and *A. flavus* by broth dilution and disk diffusion tests. These fungi commonly cause food poisoning by aflatoxin formation and can also cause aspergillosis, a severe opportunistic infection, in humans (Zhirong *et al.,* 1999). We also examined the potential synergistic effects of essential oils and synthetic antifungal drugs by combining essential oils with amphotericin B, a polyene anti-*Aspergillus* drug, which is toxic in its conventional form and very expensive in its lipidic form (Otsubo *et al.,* 1999). Additionally, the combination effects of the essential oils with ketoconazole, which has been commonly used as a broadspectrum antifungal drug, were experimentally compared.

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MATERIALS AND METHODS

Sample preparation for testing antifungal activities and fungal strains

The essential oils from *Cedrus atlantica* (wood), *Styrax tonkinensis* (wood), *Juniperus communis* (fruit), *Lavandula angustifolia* (flower), *Melaleuca alternifolia* (leaf), *Pelargonium graveolens* (leaf), *Pogestemon patchouli* (leaf) and *Rosmarinus officinalis* (herb) were extracted by steam distillation and used for evaluation of antifungal activity in this study. Citronellol, geraniol, benzoic acid, 1,8-cineol, ketoconazole and amphotericin B were purchased from Sigma Chemical Co., USA. Fungal organisms were obtained from the Korean Culture Center of Microorganisms (KCCM). *Aspergillus niger* KCCM 11239 (ATCC 9029) and *A. flavus* KCCM 11453 (YUFE 1030) were cultured in yeast and malt extract broth (YM) or malt extract liquid medium for 48 h 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).

Gas chromatography-Mass spectrometry (GC-MS)

The composition of essential oils was analyzed by GC-MS on a Hewlett-Packard 6890 GC and Hewlett-Packard 5973 MSD apparatus using a HP-5 capillary column (30 m×250 μm×0.25 μm). The temperature cycle consisted of an initial temperature of 70°C (isothermal, 5 min) that was first increased by 3°C/min to 180°C and then by 20°C/min to 270°C. The 270°C temperature (isothermal) was maintained for 10 min.

Determination of minimal inhibitory concentration (MIC)

Essential oil samples were serially diluted with 10% v/v dimethyl sulfoxide (DMSO) to obtain solutions which contained from 0.39 to 50 mg/mL essential oil, to which 10 μL Tween 80 was added. After shaking, 100 μL aliquots of the essential oil solutions were added to wells on 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 72 h. 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.

Disk diffusion assay

Fungal broth culture aliquots were added to Sabouraud dextrose agar medium and uniformly distributed. Sterile paper disks (8 mm, Advantec, Toyo Roxhi Kaisha) were impregnated with 50 μL of 25% (12.5 mg) or 50% (25.0 mg) ethanol solutions of each agent, and after alcohol evaporation the disks were placed on the culture plates. The diameter of the zone of inhibition (mm) around the disk was measured after cultivation at 26°C for 72 h. Values shown are the means of tests performed in triplicate.

Checkerboard titer test

Ten serial two-fold dilutions of geraniol, citronellol and amphotericin B were prepared using the same solvents as used in the MIC tests. 50 microliter aliquots of each geraniol dilution were added to the wells of a 96-well plate in a vertical orientation and 10 μL aliquots of each amphotericin B dilution were added in a horizontal orientation so that the plate contained various concentration combinations of the two compounds. Following this, each well was inoculated with 100 μL (ca. 5×10^4 CFU/well) of one of the two *Aspergillus* fungal suspensions and cultivated at 25°C. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of geraniol and amphotericin B, divided by the MIC of geraniol or amphotericin B alone. The FIC index, 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 to test the effect of combining the essential oil fraction from *P. graveolens* or citronellol with amphotericin B or ketoconazole.

RESULTS AND DISCUSSION

There was tremendous diversity in the composition of the tested oils. The main components of the oils are listed in Table I.

α-Cedrol (22.42%) and α-cedrene (15.68%) were major components of the oil of *C. atlantica*, one of the most prominent natural sources of antifungal agents, whereas *S. tonkinensis* oil contained relatively high amounts of benzoic acid (60.14%), 6-phenyltetrahydronaphthaline (15.76%) and benzyl benzoate (3.23%). The main components of *J. communis* were widdrene, (+)-cuparene and widdrol. 4-Terpineol was the prominent compound present in *M. alternifolia*, which has been used widely in various forms as an antiseptic and disinfectant. In the essential oil from *P. graveolens*, which is also one of the promising sources for antimicrobial drug development, citronellol (17.40%) and geraniol (5.89%) were identified as major components. The oil of *R. officinalis* contained a large amount of 1,8-cineol (19.44%), while significant quantities of valencene, guaiol and 5-azulenemethanol were identified in *P. patchouli* oil. These results suggest that there is a high potential for these oils to exhibit broad antimicrobial, including antifungal, activity.

Table I. Main components of antifungal essential oils derived from plants

Plant Name	Main Components of Essential Oils	GC Peak Area (%)
Cedus atlantica	1. α-cedrol	22.42
	2. α-cedrene	15.68
Styrax tonkinensis	1. benzoic acid	60.24
	2. 6-phenyl-tetrahydro-naphthaline	15.76
	3. benzyl benzoate	3.23
Juniperus communis	1. widdrene	25.20
	2. d-cuparene	15.34
	3. widdrol	7.74
Lavandula angustifolia	1. linalyl acetate	22.29
	2. linalol	19.19
	3. 4-terpineol	9.39
Melaleuca alternifolia	1. 4-terpineol	21.67
	2. γ-terpinene	11.84
	3. α-terpinene	9.71
Pelargonium graveolens	1. citronellol	17.20
	2. geraniol	5.89
	3. l-menthone	5.26
Pogestemon patchouli	1. valencene	6.67
	2. guaiol	5.36
	3. 5-azulenemethanol	4.60
Rosmarinus officinalis	1. 1,8-cineol	19.44
	2. camphor	12.75
	3. l-borneol	9.11

The results of the disk diffusion assays are presented in Table II. Among the tested samples at 25 mg/disk, *P. graveolens* oil (10 mm) and its main components, citronellol (12 mm) and geraniol (13 mm), most strongly inhibited *A. flavus* growth on Sabouraud dextrose agar plates. The essential oils of *M. alternifolia* and *L. angustifolia* also exhibited strong activity, but to a lesser extent than *P. graveolens* oil. It was surprising that *S. tonkinensis* and *R. officinalis*, which contain the well-known antimicrobial compounds, benzoic acid and 1,8-cineol, had relatively small inhibition zones of 5 mm at 12.5 mg/disk and 3 mm at 25 mg/disk, respectively. The oils of *C. atlantica, J. communis* and *P. patchouli* did not inhibit fungal growth. Inhibition of *A. niger* growth by the essential oils was similar to that observed against *A. flavus*. Inhibition of growth by the essential oils was dose-dependent for both fungal strains. Activities of the tested oils were dose-dependent against both fungi.

The MICs of essential oils for inhibiting *Aspergillus* growth are presented in Table III. *P. graveolens* oil exhibited the strongest activity, with MIC values of 1.56 and 0.78 mg/mL against *A. flavus and A. niger,* respectively. The *Aspergillus* species were also highly susceptible to both geraniol and citronellol. The MICs for *L. angustifolia* and *M. alternifolia* were 3.12 mg/mL against both fungi, while the essential oil from *R. officinalis* exhibited relatively moderate activity with an MIC of 12.5 mg/mL. On the

Table II. Growth inhibition by essential oils and their active components against *Aspergillus* spp., as tested by disk diffusion assay

Essential oil/Component	Inhibited Zone (mm)			
	A. flavus		*A. niger*	
	I	II	I	II
Cedus atlantica	–	–	–	–
Styrax tonkinensis	5	3	4	2
Juniperus communis	–	–	–	–
Lavandula angustifolia	6	4	5	2
Melaleuca alternifolia	9	6	8	5
Pelargonium graveolens	10	8	17	12
Pogestemon patchouli	–	–	–	–
Rosmarinus officinalis	4	2	1	–
Citronellol	12	10	19	15
Geraniol	13	10	22	18
1,8-Cineole	9	5	16	12
Benzoic acid	8	3	6	3

The values are the means of triplicate data.
I: 25 mg/disk, II: 12.5 mg/disk

Table III. MIC of essential oils and their active components against *Aspergillus spp.*

Essential Oils	MIC (mg/mL)	
	A. flavus	*A. niger*
Cedus atlantica	>25.00	>25.00
Styrax tonkinensis	0.78	0.78
Juniperus communis	>25.00	>25.00
Lavandula angustifolia	3.12	3.12
Melaleuca alternifolia	3.12	3.12
Pelargonium graveolens	1.56	0.78
Pogestemon patchouli	>25.00	>25.00
Rosmarinus officinalis	12.50	12.50
Citronellol	0.78	0.39
Geraniol	0.78	0.78
1,8-Cineole	2.50	1.25
Benzoic acid	<0.19	<0.19
Ketoconazole*	12.50	25.00
Amphotericin B*	12.50	25.00

* MIC (ug/mL)

Table IV. FIC (fractional inhibitory concentration) indices deduced from checkerboard titer tests

Samples	*A. flavus*		*A. niger*	
	Ketoconazole	Amphotericin B	Ketoconazole	Amphotericin B
Geraniol	0.520	0.625	0.625	1
Citronellol	0.525	0.562	0.625	0.625
Oil fraction of *P. graveolens*	1	0.625	0.625	0.750

FIC index = FIC of each oil (a) + FIC of ketoconazole or amphotericin B (b)

$$a = \frac{\text{MIC of the oil in combination with ketoconazole or amphotericin B}}{\text{MIC of the oil alone}}$$

$$b = \frac{\text{MIC of ketoconazole or amphotericin B in combination with the oil}}{\text{MIC of ketoconazole or amphotericin B alone}}$$

contrary, the essential oils from *C. atlantica, J. communis* and *P. patchouli* did not inhibit fungal growth, even at a concentration of 25 mg/mL. These results generally corroborate those obtained in the disk diffusion assay and also support earlier reports on the antifungal activities of herbal oils against *Aspergillus* species (Dikshit *et al.*, 1986; Mahmoud, 1994; Lis-Balchin *et al.*, 1996).

On the basis of these results, we further investigated the antifungal activity of *P. graveolens* oil and its main components, citronellol and geraniol, which are well-known antimicrobial aliphatic primary alcohols (Nidiry, 1998). Their mechanism of activity is different from that of phenolics, such as thymol or eugenol (Jayashree and Subramanyam, 1999; Voda *et al.*, 2002), and they are also advantageous in that, because of their lipophilicity, they enhance the transdermal penetration of other drugs.

The FIC indices deduced from the checkerboard titer assays, which were used to assess the effects of various combinations of geraniol, citronellol and the *P. graveolens* oil fraction with the synthetic drugs amphotericin B and ketoconazole, are listed in Table IV. Combining amphotericin B with geraniol caused a significant decrease in the MIC of each compound against *Aspergillus*, compared to their individual MIC values. For example, the MIC of amphotericin B alone against *A. flavus* was lowered from 12.5 mg/mL to 1.56 mg/mL in the presence of geraniol at 0.39 mg/mL. The MIC of geraniol alone also decreased from 0.78 mg/ml to 0.39 mg/mL when it was combined with 1.56 mg/mL amphotericin B. The FIC index calculated from these results was 0.562, which indicates the strong additive effects of geraniol and amphotericin B. When citronellol or *P. graveolens* oil was used instead of geraniol in the checkerboard titer assay, they showed similar additive effects with amphotericin B, giving an FIC index of 0.625. Combination with ketoconazole instead of amphotericin B also produced additive effects with FIC indices ranging from 0.52 to 1.00.

Thus, we have shown here that geraniol, as well as citronellol and the essential oil from *P. graveolens*, may be useful in clinical situations that require the use of amphotericin B or ketoconazole. Such combinational therapy of essential oils and synthetic antibiotics may be particularly useful against *Aspergillus species*, especially *A. flavus* (Zhirong *et al.*, 1999), a pathogenic fungus, which is one of the most common causes of invasive allergic bronchopulmonary disease in immunosuppressed and neutropenic patients. However, further investigations are required to assess the value of these natural antifungal agents for therapeutic use.

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