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Antifungal Activities of Essential Oils from Six Conifers against *Aspergillus fumigatus*[*1]

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ABSTRACT

The antifungal activities of the essential oils from *Pinus densiflora, Pinus koraiensis, Abies holophylla, Larix kaempferi, Cryptomeria japonica* and *Chamaecyparis obtusa* against *Aspergillus fumigatus* were evaluated. Essential oils were extracted from each conifer leaves for 8 h using water distillation method. The essential oil from *A. holophylla* appeared the strongest antifungal activity among the six oils with MIC value 0.125 mg/mℓ. The composition of the *A. holophylla* oil was analysed by GC/MS and then MIC was determined for main constituents of the oil. As the results, borneol and α-bisabolol, which have a hydroxyl group, showed effective antifungal activities against *A. fumigatus* (0.25 mg/mℓ). In addition, the synergistic antifungal effects were observed in the combination of borneol and α-bisabolol.

Keywords : Essential oil, conifer, antifungal activity, *Aspergillus fumigatus*, synergistic effects

1. INTRODUCTION

Essential oils are natural complex compounds characterized by a strong odour with volatility, formed by aromatic plants as secondary metabolites (Bakkali *et al*., 2008). They are obtained from flowers, buds, seeds, leaves, bark, fruits and roots as aromatic oils (Burt, 2003). They are highly volatile in the air and their fragrances differ from every species or plant materials. In nature, essential oils play an important role in the protection of the plants as antibacterials, antivirals, antifungals, insecticides and herbivores by reducing their appetite for such plants (Han, 2007; Bakkali *et al*., 2008).

Accoriding to previous researches, some essential oils showed important antifungal activity against yeasts and dermatophytes fungi which

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could predict therapeutic benefits. The properties are magnified to treat the chronic diseases such as atopic dermatitis, asthma and an allergy. Many researches are being carried out to inhibit the food and plant pathogens (*Salmonella enteritidis*, *Escherichia coli* and *Botrytis cinerea* etc.) by using antimicrobial activities of essential oils (Farag *et al*., 1989; Hammer *et al*., 1999; Muller-Riebau, *et al*., 1995; Pintore *et al*., 2002; Smith-Palmer *et al*., 1998; Wilson *et al*., 1997).

The essential oils from conifers are also applied to the cosmetics and medicines and especially good antimicrobial properties of essential oils from *Cryptomeria japonica* and *Chamaecyparis obtusa* have been reported (Cheng *et al*., 2005). In addition, *Pinus densiflora, Pinus koraiensis, Abies holophylla* and *Larix kaempferi* which are widespread in East Asia were also used along with *C. japonica* and *C. obtusa* in this experiment.

Aspergillus fumigatus plays an essential role in recycling environmental carbon and nitrogen (Latge, 1999). Its natural ecological niche is the soil, wherein it survives and grows on organic debris (Latge, 1999: Mullins *et al*., 1976). But *A. fumigatus* is opportunistic pathogen that is responsible for infections in immunocompromised patients (Hope *et al*., 2007), and *A. fumigatus* infection was reported that it commonly manifests as invasive pulmonary aspergillosis (Ben-Ami *et al*., 2010). Furthermore invasive aspergillosis causes approximately 30% of fungal infections in patients dying with cancer (Bodey *et al*., 1992).

Previous results have shown that essential oils from some plants exhibit antifungal activity against *Aspergillus* strains (Han, 2007). However activity of conifer essential oils against *A. fumigatus* have not been reviewed so much. Therefore the purpose of our research was to evaluate the antifungal activities of conifer essential oils against *A. fumigatus* and investigate action of their main compounds for the availability of conifers essential oils as forest resource in South Korea.

2. MATERIALS and METHODS

2.1. Materials

Leaves of six conifers, *P. densiflora, P. koraiensis, A. holophylla, L. kaempferi, C. japonica* and *C. obtusa* were collected in the Korea National Arboretum (KNA) in August 2011. Samples were extracted with 4 ℓ distilled water by steam distillation method at 100°C, for 8 h.

2.2. Test Organism

In this test, *A. fumigatus* (KCCM 60331) was used, and maintained on malt extract agar medium (MEA, Bacto, USA) at 26°C then kept at 4°C. Cultivated *A. fumigatus* was mixed with sterilized water to make fungal suspension. After homogenization, turbidity of the suspension was adjusted in according to McFarland standard No. 0.5.

2.3. Essential Oils Analysis

Essential oil of *A. holophylla* was analyzed by GC/MS on a Agilent 6890 GC and DB-5 column (dimension 30 m × 0.25 mm, coating thickness of 0.25 μm) and carrier gas was He. The injector and detector were adjusted to 250 and 280°C, respectively. The oven tem- perature was initiated at 50°C for 10 min, in- creased by 5°C/min to 280°C with final holding time of 10 min. Compounds were identified by comparison of each peak's mass spectra with those of authentic compounds in a mass spectra library by NIST (National Institute of Standards and Technology).

Table 1. Addition of oils to the test plates.

	1	2	3	4	5	6	7	8	9	10
mg/㎖	2	1	0.5	0.25	0.125	0.062	0.031	0.015	0.007	0.003

Table 2. MIC of essential oils of six conifers against *A. fumigatus*

MIC (mg/㎖)					
A. holophylla	*P. densiflora*	*L. kaempferi*	*P. koraiensis*	*C. japonica*	*C. obtusa*
0.125	0.25	0.5	0.5	0.5	0.5

2.4. Antifungal Activity by MIC (Minimum Inhibitory Concentration)

The effects of the essential oil from conifers and their constituents were evaluated using checkerboard titer tests (96 well). This plates were set up in accordance with the NCCLS (National Committee for Clinical Laboratory Standards) reference method. Essential oils of six conifers and constituents were prepared by two-fold serial dilution with dimethyl sulfoxide (DMSO) to final concentration from 2 to 0.003 mg/ml. Each well containing 100 ㎕ of the oil of two fold concentration (Table 1) with 10 ㎕ of *A. fumigatus* suspension. Two columns of growth controls (chemical blank) were included, with one row of the fungi blank. The plates were incubated at 26°C and were visually read after 4 days.

3. RESULTS and DISCUSSIONS

3.1. Antifungal Activities of Essential Oils from Six Conifers

The antifungal activities of essential oils from six conifers against *A. fumigatus* were shown in Table 2. MIC values were determined for *A. holophylla* (0.125 mg/㎖), *P. densiflora* (0.25 mg/㎖), and *L. kaempferi, P. koraiensis, C. japonica* and *C. obtusa* (0.5 mg/㎖), respectively. The results of this study indicated that oils of *A. holophylla* had strong effective antifungal actions against *A. fumigatus.* Result of MIC was compared with the control group after 4 days. After one week later, growth of the fungi was not observed completely. Under the test conditions as described earlier, *A. holophylla* oil showed possession activity that was more fungicidal than fungi static by *in vitro* assay.

3.2. Analysis of *A. holophylla* Essential Oil

The chemical constituents of essential oil from *A. holophylla* (AH) by GC/MS analysis were shown in Table 3. Sixteen terpene compounds were detected and those amounts were reached to 99.34%. The major constituents and their relative portions on essential oil were bornyl acetate (19.42%), limonene (16.78%), camphene (10.65%), α-pinene (10.44%), α-bisabolol (5.80%), borneol (5.37%), β-pinene (5.21%), caryophyllene (1.77%) and humulene (1.03%). And those chemical structures were shown in Fig. 1.

3.3. Antifungal Activity of the Major Constituents of AH Essential Oil

In this study, to screen for active constituent

Table 3. Chemical composition of the essential oil from *A. holophylla*

Constituent	Rt (min)	Relative portion (%)
propionic ester	11.33	2.56
α-pinene	21.42	10.44
camphene	22.20	10.65
β-pinene	23.64	5.21
3-carene	24.55	13.65
limonene	25.36	16.78
borneol	30.49	5.37
bornyl acetate	33.71	19.42
eucarvone	34.76	2.10
caryophyllene	37.56	1.77
humulene	38.49	1.03
nerolidol	40.72	1.04
caryophyllene oxide	41.74	2.74
humulene-1,2-epoxide	42.39	0.78
α-bisabolol	43.85	5.80
Unknown constituent		0.66
Total		100

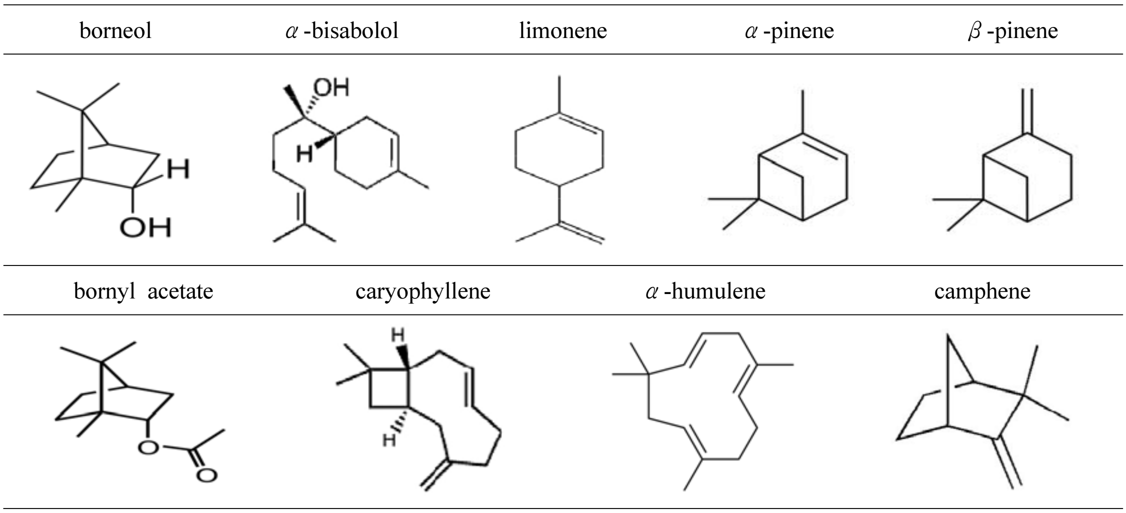

Fig. 1. Chemical structure of nine constituents from A. holophylla essential oil.

Table 4. MIC values of *A. holophylla* essential oil constituents against *A. fumigatus*

Constituent	MIC (mg/mℓ)
borneol	0.25
α-bisabolol	0.25
limonene	0.5
α-pinene	0.5
β-pinene	0.5
bornyl acetate	0.5
α-humulene	0.5
camphene	0.5
caryophyllene	0.5

Table 5. MIC values of mixed constituents, ethanol and *A. holophylla* essential oil against *A. fumigatus*

MIC (mg/mℓ)		
Mixed constituent	Ethanol	*A. holophylla*
0.125	0.5	0.125

Mixed constituent : Mixing borneol with α-bisabolol at 1 : 1 (v/v) ratio.

that appeared antifungal activity, we used the main constituents as determined by GC/MS analysis of the oil. According to Table 4, MIC values ranged from 0.25 to 0.5 mg/mℓ, and especially, borneol and α-bisabolol showed strong antifungal activity (0.25 mg/mℓ). However, the other single constituents did not show effective MIC value than crude AH oil.

The antifungal activities of each major constituent were weaker than that of the crude essential oil, because various groups of chemical compounds were contained within essential oils. It is probably that their antimicrobial activity is not attributable to one specific mechanism but there are several targets in the cell (Skandamis and Nychas, 2001; Carson *et al*., 2002). The most representative roles of chemical compounds of essential oil were to damage to cytoplasmic membrane, membrane proteins so on (Knobloch *et al*., 1989; Sikkema *et al*., 1994; Oosterhaven *et al*., 1995; Ultee *et al*., 2000; 2002; Juven *et al*., 1994). Furthermore, not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted (Burt, 2003). Another research reported that the crude essential oils appeared more positive effect than separating their compounds, because combination of various chemicals in essential oils lead to synergistic or antagonistic effects (Delaquis *et al*., 2002).

Borneol and α-bisabolol were known as the terpene that containg a hydroxyl group. This fact led that the presence of the hydroxyl group in the constituents of essential oil affected the antifungal activity. The importance of the phenolic hydroxyl groups for the antimicrobial activity of the monoterpenes has previously been reported (Dorman *et al*., 2000; Ultee *et al*., 2002).

3.4. Antifungal Activity of Combined Constituents

To identify synergism of two alcohol group containing compounds to enhance the efficacy, checkerboard assays of mixed constituent, ethanol and AH oil were carried out. As shown in Table 5, the MIC of borneol combined with α-bisabolol against *A. fumigatus* was decreased to 0.125 mg/mℓ. The MIC value of combined constituent was lower than each single constituents of AH oil.

To enhance the efficacy of essential oils, the combined use of different oils has been evaluated recently for potential synergistic effects. In this study, we observe a similar effect from crude AH essential oil of antifungal activity by mixing borneol and α-bisabolol with the ratio of 1 : 1. According to the results, we assumed that the combination of phenolic alcohol group containing constituents will probably results in a more strong antifungal activity.

According to previous researches, it is difficult to know what constituents of essential oils affecting the antifungal activities accurately. In other words, the mechanism of action by the antimicrobial properties of essential oils and their components have not been studied in great detail (Lambert *et al*., 2001). In this study, it is supposed that the antifungal activity of AH oil against *A. fumigatus* can be related to the presence of borneol and α-bisabolol which is contained phenolic hydroxyl group.

MIC was evaluated using ethanol against *A. fumigatus* to figure out how the presence of hydroxyl group influenced the antifungal activity. But the activity of ethanol with MIC value 0.5 mg/㎖ was much inferior to the mixture of borneol and α-bisabolol or crude essential oil from AH. This results showed that the hydroxyl group in constituents of essential oil was not mandatory for antifungal activity. Based on these data, the phenolic ring structures in monoterpene compounds affected the antifungal activity instead of hydroxyl group. However it is not reasonable assumption, because of considering a study on the antimicrobial activity using a linalool (Alviano *et al*., 2005). Thus, it is assumed that both hydroxyl group in constituent of essential oil and a unique chemical structures of each constituent contributed to good antifungal activity of borneol, α-bisabolol and their mixture.

4. CONCLUSION

In this study, the antifungal activities of essential oils from *A. holophylla, P. densiflora, L. kaempferi, P. koraiensis, C. japonica* and *C. obtusa* were examined. Among the oils, *A. holophylla* showed the strongest antifungal action against *A. fumigatus.*

According to the analysis of chemical compounds in essential oil from *A. holophylla* by GC/MS, major constituents were selected to examine MIC values. As a result, borneol and α-bisabolol showed the lowest MIC value. Therefore the strong effect of AH essential oil was appeared due to the content of borneol and α-bisabolol defined as strong antifungal agent. Although a positive effect against *A. fumigatus* was appeared from two constituents, it was not indispensable factor to antifungal activity. However the positive effect of borneol and α-bisabolol was may explained by their chemotype with hydroxyl group in structure. Interestingly, the antifungal activity of the combination of borneol and α-bisabolol was similar to crude essential oil from *A. holophylla*. This meant that the mixture of some constituents also could obtain a strong activity against fungi, although antifungal activities of essential oils were dependent on a synergistic effect of their a number of constituents.

In conclusion, this study suggested that the possibility on utilization of essential oils from conifers as antifungal agent. Further studies about a side effect or stability in the body of essential oils are necessary for pharmaceutical purpose.

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