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Cytyotoxicity and Anti-*Malassezia* Activity of Limonene

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A previous study of ours indicated that *Citrus auranifoli* oil possesses antifungal activity against *Malassezia furfur* and *Malassezia pachydermatis*. In this study, we evaluated the anti-*M. furfur* and *M. pachydermatis* activities of limonene, which is a major component of *C. aurantifolia* oil, using the disk diffusion method. We also examined cytotoxicity against human normal epithelial (Beas-2B) cells using the cytopathic effect reduction (CPE) method. The results revealed that the minimum fungicidal concentration (MFC) value of limonene is lower than the value for itraconazole. The MFC value of limonene was seen to be 7.81 μg/mL against *M. furfur* and 3.90 μg/mL against *M. pachydermatis*. MFC values of itraconazole against *M. furfur* and *M. pachydermatis* were 62.50 μg/mL and 31.25 μg/mL, respectively. In addition, it was noted that limonene was not toxic to Beas-2B cells with normal morphology at a concentration of 100 μg/mL. However, itraconazole exhibited weak toxicity at the same concentration. Therefore, our results indicate that limonene could potentially be effective at controlling *M. furfur* and *M. pachydermatis* infections with no cytotoxicity.

Keywords: Anti-*Malassezia,* cytotoxicity, limonene

Malassezia species are associated with a number of dermatological disorders [1]. Azole drugs such as fluconazole and ketoconazole are used for treatment of such illnesses [5]. However, these agents include severe toxic hepatitis, acquired cutaneous adherence [7, 9]. The development of safe, effective, and inexpensive antifungal agents is among top global priorities in drug development. In many years, there are a few reports concerning the susceptibility of *Malassezia* to natural antifungal agents. Therefore, the development of more commercial antifungal agents is required.

Our previous study has been shown that *Citrus aurantifolia* oil among many plant essential oils possessed strong anti-activity *M. furfur* and *M. pachydermatis* [3, 4]. In this article, we evaluated inhibitory effect of limonene against *M. furfur* and *M. pachydermatis* which is a major component of *C. aurantifolia* oil against the two fungi and cytotoxicity against Beas-2B (human normal epithelial) cell. The limonene was obtained from Dr. Y.J. Ahn of Seoul National University.

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In our experiments, itraconazole and 1-methyl-4-(prop-1-en-2-yl)-cyclohexene (lemonene) was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in ethyl alcohol, and stored at -20°C.

M. furfur (KCCM 12679) and *M. pachydermatis* (KCCM 50374) were obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). Condition and media to grow *M. furfur* and *M. pachydermatis*, and method to test anti-fungal activity followed by a previous report [3].

To evaluate the minimum fungicidal concentration (MFC), limonene concentrations in solution with 95% ethanol ranging from 0.69 to 500 μg/mL, together with a negative control disk (only ethanol treated group) and a itraconazole (positive control) disk were tested. Inocula were prepared with fungal saline suspension, with a cell concentration between 0.40 and 0.49 of absorbance for bacterial inoculum, and between 0.60 and 0.69 for the fungi inoculum. The spectrophotometer readings were taken at a wavelength of 600 nm; these procedures created suspensions with a concentration of approximately 1.0×10^6 CFU/mL. The CLSI M27-A3 (2008) protocols were used to obtain the MFC [10]. The time of contact among the microorganisms and essential oils was 24 h. The innocuity of the 95% ethanol was evaluated by diluting it to 3% in medium 37 broth or

sabouraud dextrose broth (SDB). The positive and negative controls were tested at the same concentration. The controls for inoculum viability and growth medium sterility were formulated according to the above protocols.

Beas-2B cells were purchased from American Type Culture Collection (Rockville, MD) and were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic solution at 5% CO_2 incubator of 37°C. Antibiotic-antimycotic solution, trypsin-EDTA, FBS and MEM were supplied by Gibco BRL (Grand Island, NY, USA). Sulforhodamine B (SRB) was purchased Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade and obtained from Burdick and Jackson (Muskegon, MI, USA). The cytotoxicity of limonene against Beas-2B cell was determined by cytopathic effect reduction method, the morphology of cells was observed under microscope of 32 × 10 magnifications (AXIOVERT10, ZEISS, Germany), and images were recorded [2].

In the results, we found MFC of 7.81 μg/mL for limonene against *M. furfur* and 3.90 μg/mL for limonene against *M. pachydermatis.* MFC values of itraconazole against *M. furfur* and *M. pachydermatis* were 62.50 μg/mL, 31.25 μg/mL, respectively (Table 1). Also, limonene wasn't toxic to Beas-2B cells with typical spread-out shapes and normal morphology at a concentration of 100 μg/mL (Fig. 1). However, itraconazole showed weak toxicity at same concentration with cell viability of 90.3% (Fig. 1).

Table 1. Minimum fungicidal concentration (MFC) of limonene against *M. furfur* and *M. pachydermatis*

Strains	MFC (μg/mL)	
	Limonene	Itraconazole
M. furfur	7.81	62.50
M. pachydermatis	3.90	31.25

There have been studies supporting antibacterial activity in various *Citrus* species and its components but studies evaluating *M. furfur and M. pachydermatis* effect of limonene have not been reported [6, 8, 11].

In our study, *C. auranifoli* oil showed an inhibitory effect against *M. furfur* and *M. pachydermatis* and the main constituent of *C. auranifoli* oil by GC-MS analysis was limonene [4, 5]. In this study, limonene had some higher antifunagal activity than that of itraconazole showing low MFC values against two strains.

Consequently, we demonstrated that the limonene had an inhibitory activity against *M. furfur and M. pachydermatis* with lower MFC value than itraconazole. Also, limonene showed noncytotoxicity on Beas-2B cells with normal morphology. Therefore, it can be concluded that limonene

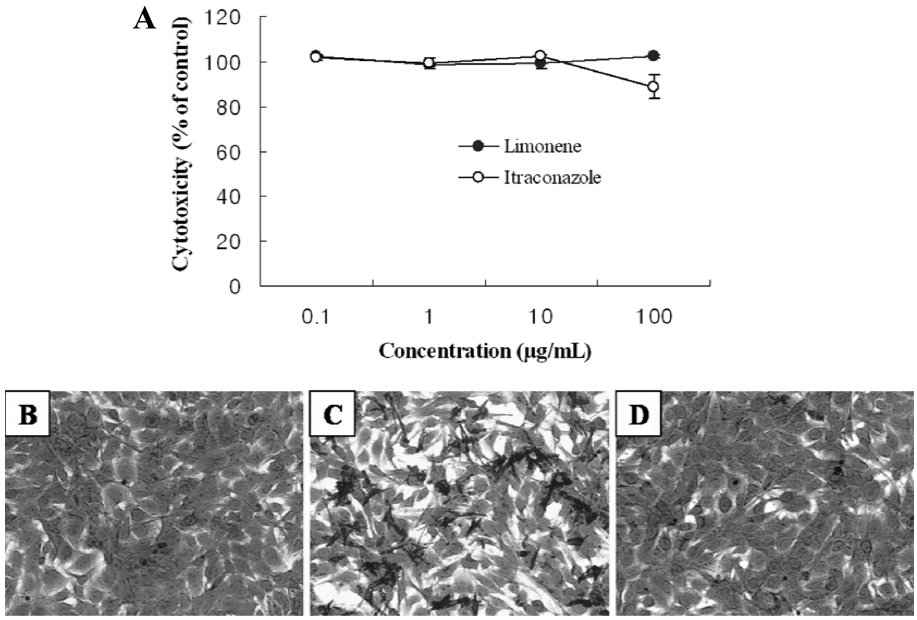

Fig. 1. Cell viability of Beas-2B cells treated with limonene. Hundreds μg/mL of each extract was added to Beas-2B cells. After 1 day, cell viability was evaluated by SRB method and the morphology of cells was photographed under a microscope [2]. Each value is the result of mean ± S.D. of three independent experiments. (A) Cell viability of Beas-2B cells after treatment of 100 μg/mL limonene; (B) Non-treated cells; (C) Cells treated with itraconazole; (D) Cells treated with limonene.

may have interesting applications on diseases caused from *M. furfur and M. pachydermatis.*

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국문초록

Limonene의 세포독성과 항*Malassezia* 활성

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우리의 이전의 연구에서 *Citrus auranifoli* 오일은 *Malassezia furfur*와 *Malassezia pachydermatis*에 대해 항곰팡이 활성을 나타낸다는 것을 보여주었다. 본 연구에서 *Citrus auranifoli* 오일의 주요 성분인 리모넨(limonene)의 *M. furfur* 와 *M. pachydermatis*에 대한 저해효과를 디크스 확산 방법(disk diffusion method)에 의해 측정하였고, Beas-2B 세포에 대한 세포독성을 cytopathic effect reduction 방법에 의해 측정하였다. Limonene의 최소곰팡이저해농도(minimum fungicidal concentration)는 양성대조구인 itraconazole보다 더 낮았고, 100 μg/mL의 농도에서 Beas-2B cells에 대한 세포독성을 나타내지 않았다. 그러나 itraconazole은 같은 농도에서 약한 세포독성을 나타내었다. 그러므로, 우리의 결과에서 limonene는 비세포독성과 함께 *M. furfur* and *M. pachydermatis*의 성장저해를 하는데 효과적이라는 것을 보여준다.