Antifungal Effect of Eugenol and Nerolidol against Microsporum gypseum in a Guinea Pig Model

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Essential oils have been widely used in anti-infectious application. In the present study, we elucidated the antifungal activities of eugenol and nerolidol isolated from Japanese cypress oil in a guinea pig model infected by Microsporum gypseum (M. gypseum). A minimal inhibitory concentration (MIC), skin lesion scoring, hair culture and histopathologic examination of skin tissues were performed to evaluate the antifungal effect of these oils. The MICs of eugenol, nerolidol and econazole (positive control) were 0.01–0.03% and 0.5–2% and 4–16 μg/ml, respectively. Based on these MICs, eugenol and nerolidol were adjusted to 10% concentration with a base of Vaseline® petroleum jelly and were applied topically to the skin lesion infected with M. gypseum daily for 3 weeks. Both eugenol and nerolidol were clinically effective at improving the lesion during the first week of application, as determined by skin lesion scoring. Nerolidol improved the skin lesions infected by M. gypseum, but eugenol did not, as determined in the hair culture test. Histopathologic examination revealed that the eugenol-and nerolidol-treated groups had a lower degree of hyperkeratosis and inflammatory cell infiltration than the positive control. Taken together, these results suggest that eugenol and nerolidol could apply supplementary antifungal agents.

Key words eugenol; nerolidol; antifungal activity; Microsporum gypseum; guinea pig

Dermatophytosis is a superficial infection caused by keratinophilic fungi, known as dermatophytes, in keratinized tissues including hair, nail and stratum corneum of skin. The dermatophytes include three genera: Microsporum, Trichophyton and Epidermophyton. The species that most commonly infect animals are Microsporum canis, M. gypseum and Trichophyton mentagrophytes. The fungi invade into keratinized tissues and hair follicles, and cause patchy alopecia, scale, and subsequent inflammation.1,2) Traditional antifungal drugs have variable adverse effects, and fungal resistance is becoming more common. Thus, more potent and safer antifungal agents need to be developed.3–5) Various plant materials are believed to have antifungal activity, and many essential oils have been reported to have antifungal activities.6–8) Japanese cypress (Chamaecyparis obtusa) is an evergreen tree in the Cupressaceae family with antibacterial and antifungal activities as well as a sedative, stress-reducing effect which decreases serum cortisol levels.9,10) Eugenol and nerolidol are extracted from an essential oil from Japanese cypress. Eugenol is a major phenolic component of clove oil used in dentistry as a root canal sealer. Eugenol has been reported to inhibit the growth of bacteria, including oral bacteria11) and some gram positive and negative bacteria (Escherichia coli (E. coli), Staphylococcus aureus (S. aureus), Listeria monocytogenes (L. monocytogenes), Lactobacillus sakei) by interaction with the cell membrane.12,13) Antifungal activities of Eugenol have also been described in vitro and in vivo.14,15) Silva et al. demonstrated that eugenol, a main constituent of essential oils of Ocimum gratissimum, has antidermatophytic activities in vitro; this effect has not been confirmed in vivo. Nerolidol is a sesquiterpenoid component of essential oil used to enhance flavor and aroma, and has been studied as a topical skin penetration enhancer.16–18) In addition, nerolidol has inhibitory activities on S. aureus and E. coli by altering bacterial cell permeability,19,20) but it has not been evaluated for antifungal activities.

Thus, in the present study, antifungal activities of the essential oils, eugenol and nerolidol, were evaluated against dermatophytes. The antifungal activities of these biomaterials were assessed against one dermatophyte, M. gypseum, using the microdilution method in a guinea pig model.21,22)

MATERIALS AND METHODS

Experimental Animals In the present study, 20 male albino guinea pigs (5-week old, 350 to 400 g) were obtained from Dae Han Biolink Co., Ltd. (Eumsung, Chungbuk, Korea). All animals were individually housed in polycarbonate cages, and used after acclimation to an environmentally controlled room (temperature: 23±2°C, relative humidity: 50±10%, frequent ventilation and 12 h light cycle). All experimental and animal procedures were approved by the Ethics Committee of the Chungbuk National University.

Test Materials Eugenol and nerolidol were extracted from Japanese cypress at the Department of Forest Products College of Agriculture and Life Sciences, Seoul National University (Seoul, Korea). Econazole nitrate, as a positive control, was purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.).

Test Organism M. gypseum was isolated from a guinea pig infected with dermatophytosis at the Veterinary Medical Center of Chungbuk National University (Cheongju, Korea) and microscopically identified based on the morphology of macroconidia. The organisms were cultured on Sabouraud dextrose agar plates (Becton Dickinson, Sparks, MD, U.S.A.) at 30°C for 7 to 10 d. Fungal conidia were harvested by gen-
ly scraping them off with sterile saline containing 0.1% tween-80. The suspension was filtered through sterile gauze to remove large hyphae. The fungal conidia were counted with a hemocytometer.

**Minimal Inhibitory Concentration (MIC) Determination**

The standard broth microdilution method for econazole was applied according to the National Committee for Clinical Laboratory Standards guideline (NCCLS, 1998). Econazole stock solution (12800 μg/ml in dimethylsulfoxide) was diluted by RPMI 1640 medium (with 2% l-glutamine, without bicarbonate) buffered to pH 7.0 with 0.165 M morpholinosulfonic acid (MOPS). The final fungal conidial concentration was adjusted to 5×10^3 conidia/ml, corresponding to 0.4—5×10^4 CFU/ml, as recommended by NCCLS. The drug solution and conidial suspension were dispensed into 96-well microplate wells with 100 μl aliquots, respectively. Final concentrations of econazole were 0.25 to 128 μg/ml. The plate was incubated at 30 °C and observed daily for fungal growth. The MIC was visually determined as the lowest concentration to inhibit fungal growth at 7 d from incubation. The MICs of eugenol and nerolidol were determined by modifying the NCCLS guideline. To increase the solubility, these materials were diluted two-fold with sterile saline containing 10% tween-80. The stock concentration of eugenol and nerolidol was 2% (v/v). Conidial suspension was prepared with RPMI-1640 medium buffered with MOPS. The final fungal conidial concentration was adjusted to 5×10^3 conidia/ml. The same procedures were used as prepare econazole. The final concentration of Eugenol and nerolidol was 0.01 to 2%.

**Animal Infection**

An animal model for infection was chosen based on previous studies. The inoculum concentration of *M. gypseum* conidia was 1×10^7 conidia/ml. Before inoculation, the hairs of both flanks of each guinea pig were widely plucked manually, and the hairless skin was slightly abraded with a blade. One hundred microliters of inoculum (1×10^6 conidia) was inoculated on the skin encircled by an open glass cylinder (2.5 cm×2.5 cm). The inoculation area was fixed to the flank by bandaging, which was maintained during the entire experimental period (8—15 d after treatment). Hair samples from inoculated skin were plucked and put onto Sabouraud dextrose agar for fungal growth. Upon identification of a fungal colony, the sample was considered positive. The number of positive hair samples per group was expressed as a percentage, or culture positive rate (%). One week after final drug application, all animals were euthanized, using ether, for skin biopsy. From a skin punch biopsy of 6 mm in diameter, all mycotic skin lesions were excised regularly. These skin tissues were fixed in 10% neutral buffered formalin and processed for histopathologic examination. Each tissue section was stained with hematoxylin and eosin, and topical antifungal activities of eugenol and nerolidol were evaluated.

**Data Analyses**

The skin lesion scores were analyzed by the Kruskall–Wallis test followed by the Mann–Whitney U-test (with SPSS for Windows). p values of less than 0.05 were regarded as significant.

**RESULTS**

**MIC**

Table 1 indicates the MICs of eugenol, nerolidol and econazole to *M. gypseum*. The MICs of eugenol and nerolidol were 0.01—0.03% and 0.5—2%, respectively. The MIC of econazole was about 50 times lower than that of nerolidol.

**Skin Lesion Scoring**

Changes in gross findings of skin lesions and skin lesion scores following drug treatment are shown in Figs. 1 and 2, respectively. Most infected skin lesions showed similar well-defined redness and scaling, corresponding to lesion score 3, on the first day of drug treatment. In contrast, the lesion scores of the positive control were maintained until 8 d post-treatment, while scores of the treated groups continuously decreased following topical treatment. Eugenol- and nerolidol-treated groups had significantly lower lesion scores (p<0.05) than the positive control. As more time passed, however, no significant reduction in lesion scores was observed in these groups compared to the positive control. The nerolidol-treated group had a lower lesion score than the eugenol-treated group. Econazole significantly reduced lesion scores compared to the positive control during the entire experimental period (8—15 d after treatment: p<0.01, 22 d after treatment: p<0.05).

**Hair Culture**

Changes in hair culture positive rates following drug treatment are shown in Table 2. At day 1, all hairs of inoculation areas were positive on culture medium. Including the positive control, the culture positive percentage of each experimental group was gradually reduced in a time-dependent manner. The hairs of the econazole-treated group did not produce a fungal colony after day 8 of treatment. The hair culture positive rates of the eugenol-treated group did not produce a fungal colony after day 8 of treatment.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>MIC</th>
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<tbody>
<tr>
<td>Eugenol</td>
<td>0.01—0.03%</td>
</tr>
<tr>
<td>Nerolidol</td>
<td>0.5—2%</td>
</tr>
<tr>
<td>Econazole</td>
<td>4—16 μg/ml (0.0004—0.0016%)</td>
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</table>
not decrease, while nerolidol continuously decreased the rate as compared to the positive control.

**Histopathologic Finding**  Histopathologic examination was performed to identify any fungal hyphae or pathologic changes in inoculated skin (Fig. 3). Fungal hyphae were not identified in any of the groups. Hyperkeratosis, pustules, hair folliculitis and infiltration of inflammatory cells into the dermis layer were observed in the positive control. In the eugenol-, nerolidol- and econazole-treated groups, a lower degree of hyperkeratosis and inflammatory cell infiltration was observed compared to the positive control.

**DISCUSSION**

Various essential oils have been used clinically for antifungal and anti-bacterial effects. In this study, we evaluated the antifungal activities of eugenol and nerolidol in an animal model. The agar gel diffusion and broth dilution method have been used for antifungal susceptibility testing. The disc diffusion method is simple, convenient and has been used for

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**Table 2. Time-Course Changes in Hair Culture Positive Rates in Guinea Pigs Infected with *M. gypseum* **

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
<th>Day 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0/8 (0)</td>
<td>0/8 (0)</td>
<td>0/8 (0)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>PC</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
<td>6/6 (83.3)</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
<td>6/6 (100)</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>Nerolidol</td>
<td>5/5 (100)</td>
<td>4/5 (80)</td>
<td>3/5 (60)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Econazole</td>
<td>5/5 (100)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

Hair culture positive rate indicates the identification of a fungal colony following hair culture on Sabouraud dextrose agar. PC, positive control; NC, negative control.
antifungal susceptibility testing of essential oils.\textsuperscript{14,16} In addition, Karaca and Koc reported that the disc diffusion method for susceptibility testing of dermatophytes was consistent with the dilution method.\textsuperscript{21} However, the zone diameter from this method was affected by fungal concentration and the degree of drug diffusion into the agar. We adopted the standard broth dilution method for filamentous fungi established by the NCCLS.\textsuperscript{24} This method appears to be the method most commonly used for antifungal susceptibility testing of tea tree oil.\textsuperscript{7}

In this study, fungal MICs were determined by the NCCLS guideline. The final fungal conidial concentration was adjusted to $5 \times 10^{4}$ to correspond to the fungal concentration suggested by NCCLS. Compared to the powdery forms of antifungal agents used in NCCLS, oil agents used in this study are less soluble in the culture medium. To increase solubility, the final concentration of the detergent tween-80 was increased to 5%. This solvent enables dissolution of the oils up to 4% concentration in the medium. Remmal et al. suggested to employ agar solution to disperse oils because detergents inhibit the antimicrobial activities.\textsuperscript{25} However, Hammer et al. reported that the antifungal activity of tea tree oil was not affected by increased detergent concentration.\textsuperscript{26}

The MIC of eugenol against \textit{M. gypseum} was 0.01—0.03%, and at higher concentrations eugenol had fungicidal activities. Silva et al. showed that \textit{M. gypseum} was completely inhibited by eugenol at a concentration of 250 \(\mu\)g/ml (similar to the concentration used in this study) in the agar gel diffusion method.\textsuperscript{16} Eugenol reportedly induces potassium leakage from \textit{E. coli} and \textit{S. aureus} and inhibits energy uptake or utilization in \textit{L. monocytogenes}.\textsuperscript{8,12} Eugenol can damage envelopment of Candida by leaking substances with absorbance at 280 nm.\textsuperscript{15} In this study, it is supposed that this membrane interaction of eugenol causes antifungal activities against \textit{M. gypseum}.

Nerolidol was demonstrated to have antifungal activities against \textit{M. gypseum}, with 0.5—2% MICs, although it is less potent than eugenol. However, nerolidol more effectively reduced the skin lesion score and culture positive rates than eugenol. The antifungal activity of nerolidol has been demonstrated against dermatophytes. Nerolidol, a lipophilic terpene, also has antibacterial activity and enhances other antibiotic drug actions by increasing bacterial permeability or susceptibility to drugs.\textsuperscript{19} Similar to eugenol, nerolidol disrupts normal bacterial membrane barrier function by leaking potassium ion from \textit{S. aureus}.\textsuperscript{20} Nerolidol enhances topical drug permeability by increasing drug diffusion and disrupting highly organized packing of stratum corneum.\textsuperscript{17,18} Thus, nerolidol may have potent antifungal activity when administered as combination therapy with other antifungals.

The proliferation phase of \textit{M. gypseum} in experimentally infected guinea pigs lasts 5 to 11 d followed by a spontaneous healing period up to 26 d after infection.\textsuperscript{22} In this study, the experimental therapeutics was topically applied after 5 d of infection for 26 d. Eugenol and nerolidol significantly reduced the skin lesions during the first week of drug treatment during the fungal proliferation period. This result indicates that eugenol and nerolidol effectively induce clinical remission, especially during fungal proliferation. Econazole showed more potent antifungal activities than eugenol and nerolidol, but the histopathologic findings associated with all three treatments did not differ. In addition to the antifungal effect, other complex activities including antibacterial and stress-reducing activities of these oils may contribute to gross lesion remission and histopathologic changes in infected skin. Furthermore, the topical application of nerolidol may improve the disruption of the skin stratum corneum to aid lesion remission. The distinctly different results of these essential oils in \textit{in vitro} and \textit{in vivo} systems warrant to confirm antifungal activities in \textit{in vivo} tests.

Eugenol and nerolidol effectively induced clinical lesion remission during the early stage of healing. The hair culture experiment showed that nerolidol more effectively improved the infected skin lesions than eugenol. The degree of hyperkeratosis and inflammatory cell infiltration was less in animals treated with the oils than in the non-treated animals. Taken together, these results suggest that eugenol, and in particular nerolidol, could be effective supplemental topical antifungal drugs for clinical remission of dermatophytosis.

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\section*{REFERENCES}