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In vitro Study and Clinical Trial of Natural Essential Oils and Extract Against *Malassezia* Species

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Background: *Malassezia*, a lipophilic yeast, is a causative agent for dandruff and seborrheic dermatitis. Many biological agents have been studied for anti-*Malassezia* effect but further studies are needed for their clinical application.

Objective: The study was conducted to evaluate the inhibitory effect of different natural essential oils and a fruit extract on *Malassezia* species in an *in vitro* study and a clinical trial.

Methods: The antifungal effects of natural essential oils and a fruit extract on *Malassezia* species (*M. furfur* and *M. sympodialis*) were evaluated by measuring the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) and using the disc diffusion method. Natural essential oils of citron seed, lavender, and rosemary and citrus junos fruit extract were used for the *in vitro* study. The clinical trial was conducted with a shampoo containing four ingredients. A total of 22 subjects used the shampoo every day for 4 weeks and were evaluated using clinical photography, trichoscopy, and sebumeter at baseline, 2 weeks, and 4 weeks after treatment.

Results: Antifungal activity of agents was relatively lower in lavender and rosemary essential oils at MIC and MFC. Disc diffusion method revealed same results. In the clinical trial, the amount of sebum decreased statistically significantly and erythema, dandruff, and lesion extent also improved.

Conclusion: The natural essential oils and fruit extract are effective for suppressing *Malassezia* activity, therefore these might be used as an alternative for treatment of dandruff and seborrheic dermatitis.

Key Words: Citron, Dandruff, Lavender oil, *Malassezia*, Rosemary oil, Seborrheic dermatitis

INTRODUCTION

Seborrheic dermatitis and dandruff are common chronic inflammatory disorders mainly affecting the face, scalp, or upper trunk but the exact etiology is not fully understood. Pathogenesis of seborrheic dermatitis and dandruff is usually explained as *Malassezia* colonization, increased sebum secretion, and individual susceptibility, which includes genetics, emotional stress, and immunity. Treatment focuses on controlling symptoms rather than curing the disease[1-4].

Malassezia (M.) species comprise part of the normal flora of the skin, however, they have been implicated in pityriasis versicolor, seborrheic dermatitis, atopic dermatitis, psoriasis, and pityrosporum folliculitis[5]. Guého et al.[6] classified *Malassezia* genus into 7 species: *M. globosa*; *M. restricta*; *M. obtusa*; *M. slooffiae*; *M sympodialis*; *M. furfur*; and *M. pachydermatis* in

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1996. To date, 15 species have been reported[7].

Various natural essential oils or extracts are already well known for their health benefits. Citrus fruits are rich in antioxidant phenols and contain many flavonoids showing anti-inflammatory effect[8-11]. Lavender essential oils are well known for ameliorating anxiety and depression, their general use for aromatherapy as well as anti-oxidant, and anti-infectious effects[12-15]. Rosemary essential oils are also popularly used for antioxidant, antimicrobial, and anti-inflammatory effect[16-18].

In this study, we purposed to evaluate the antimicrobial effects of natural essential oils and fruit extract on *Malassezia* species *in vitro*. In addition, clinical trial with shampoo containing citrus junos fruit extract and citron, lavender, and rosemary essential oils was conducted.

MATERIALS AND METHODS

1. Natural essential oils and fruit extract

Three kinds of essential oils, lavender, rosemary, and citron seed essential oils were used in this study. In addition, citrus junos fruit extract was used. Lavender and rosemary essential oils were obtained from Namwon Jeollabuk-do, South Korea and extracted at HISOL Co., Ltd (Korea). Citron seed essential oil and citrus junos fruit extract were obtained from Goheung Jeollanam-do, South Korea and extracted at HISOL Co., Ltd (Korea). The positive control substance was zinc pyrithione (Sigma-Aldrich, St. Louis, Missouri, USA), which has antimicrobial effect on dandruff and seborrheic dermatitis.

2. Culture of *Malassezia* species

Malassezia furfur (*M. furfur*, CBS 1878) and *Malassezia sympodialis* (*M. sympodialis*, CBS 7977) were cultured using modified Leeming & Notman's broth I[19] (mLNA broth I: glucose 20 g, malt extract 50 g, polypeptone 1 g, oxgall 20 g, tween 40 0.1 g, glycerol 0.02 g, agar 15 g, and dextrose in water 1,000 mL) at 35℃ in an orbital incubator (IS-971R, Lab companion, Korea).

3. Quantitative evaluation of anti-*Malassezia* activity (Minimal inhibitory and minimal fungicidal concentration)

The principle of the test was modified from reference method M27-A3 (CLSI, 2008) of the Clinical and Laboratory Standards Institutes (CLSI)[20,21]. Essential oils or fruit extract were serially diluted two-fold in broth medium[19]. The final concentration of oils in the medium ranged from 1~0.0039% (v/v), in a sterile broth medium in test tubes. *Malassezia* species culture, 0.5 McFarland standard (Eucast, 2003), was inoculated into test tubes containing 2 mL of the various concentrations of the agents in the broth medium. The samples were incubated at 35℃ for 48 h and thereafter observed for growth or turbidity. After incubation, the last tube without any visible growth of the fungus was taken to represent the minimal inhibitory concentration. All samples showing no turbidity were subcultured and the lowest concentration from which the microorganisms did not recover, was the minimal fungicidal concentration. The minimal inhibitory concentration was defined as the lowest concentration of essential oils or active compound inhibiting visible growth of the fungus and was determined to have an inhibition rate of fifty percent minimal inhibitory concentration (MIC_{50}) after the incubation. The minimal fungicidal concentration was defined as the lowest concentration of essential oils or active compound in the test tube from which the microorganisms did not recover, and was determined as the "no colony" concentration in the agar medium[19]. Control samples (positive and negative) were incubated under the same conditions. MIC/MFC testing was carried out at least three times.

4. Disc diffusion method

The antifungal activity of the essential oils was assessed by a minor modification of the previous method using 100 μL of suspension containing 5×10^6 CFU/mL of *Malassezia* species[19,22]. The discs (Whatman, 6 mm in diameter) were impregnated with 20 μL of essential oil diluted under aseptic conditions and placed on the inoculated agar[23]. Negative controls were prepared using the same solvent that was spread on the agar plates. The antifungal activity was evaluated by measuring the inhibition zone diameter observed after 48 h of incubation.

5. Clinical trial with shampoo containing natural oils and fruit extract

This therapeutic study was approved by the Institutional Review Board of the Chonnam National University Hospital (IRB no. CNUH 2017-287). All the subjects signed informed consent prior to enrollment. Patients with dandruff and/or seborrheic dermatitis on the scalp, aged from 19 to 54 years were included in this study. Patients with other chronic and inflammatory dermatoses and/or alopecia on the scalp, those who had been taking any other oral medications (including oral steroid, finasteride, dutasteride, antihypertensive agents,

Table 1. The minimal inhibitory concentration $(MIC)_{50}$ of essential oils and fruit extract

Variables	*M. furfur* (%)	*M. sympodialis* (%)
Lavender oil	0.125	0.0625
Rosemary oil	0.25	0.125
Citron seed oil	–	–
Citrus junos fruit extract	–	–
Zinc pyrithione Suspension (control)	0.000625	0.000039

–: no activity under 1%

cyclosporine, spironolactone, and cimetidine) that can influence the result, those who had been using topical steroid or antifungal shampoo 1 month earlier, were excluded. All the patients were informed to massage their scalp for 5 minutes with the shampoo containing natural oils and fruit extract and then rinse with water, daily at the morning for 4 weeks.

The shampoo containing three kinds of essential oils (lavender, rosemary, and citron seed essential oils) and one fruit extract (citrus junos fruit extract) was used in this study; the shampoo was made at HISOL Co., Ltd (Korea). The shampoo contained 2% of fruit extract or oils, respectively.

Three clinical parameters (erythema, scaling, and lesion extent) were measured by the same investigator using a 4-point scale (0 = none, 1 = mild, 2 = moderate, 3 = severe)[24]. Erythema was measured using a 4-point scale which 0 point meant no involvement, 1 point meant light pink color, 2 point meant pink color and 3 point meant marked red color. Scaling was measured using a 4-point scale which 0 point meant no involvement, and 3 point meant thick yellowish confluent plaque or sheet. The lesion extent was measured using a 4-point scale which 0 point meant no involvement, 1 point meant less than 30% involvement, and 3 point meant more than 70% involvement. The sebum secretion at the vertex, frontal, right temporal, and left temporal areas of the scalp was measured using a Sebumeter (Courage & Khazaka Electronic, Cologne, Germany). Clinical photographs using camera (Canon EOS 40D, Canon Inc., Japan) and trichoscopic examination using a dermoscope (Dermlite II Pro; 3Gen, California, USA) attached to a digital camera (Nikon Coolpix P6000, Tokyo, Japan) were also taken. All the measurements were assessed at baseline, 2 weeks, and 4 weeks after using the shampoo (3 hours after the latest washing). This assessment was conducted in a room maintained at humidity of 40%±2% and temperature of 22~24℃.

6. Statistical analysis

All *in vitro* values for statistical analyses were performed using one-way analysis of variance (ANOVA) with a post hoc Least Significant Difference (LSD) test. In the clinical trial, Mann-Whitney test and Wilcoxon signed rank test were used for comparing the sebum secretion, erythema, dandruff, and lesion extent of the subjects.

All values are expressed as the mean ± S.D. All analysis was performed using SPSS version 23.0 (SPSS, Chicago, IL). A p-value <0.05 was considered statistically significant.

RESULTS

1. Minimum inhibitory concentration

Antifungal activity of four agents was measured against two *Malassezia* species compared with zinc pyrithione, a positive control agent. The results are summarized in Table 1. Among four agents, only two essential oils showed antifungal activity under 1% with MIC_{50} ranging from 0.0625% to 0.25%. MIC_{50} against *M. furfur* and *M. sympodialis* was lowest in lavender oil showing 0.125% and 0.0625%, respectively. Citron seed oil and Citrus Junos fruit extract failed to inhibit *M. furfur* and *M. sympodialis* under 1%. Two essential oils were more effective in inhibiting *M. sympodialis* compared to *M. furfur*.

2. Minimal fungicidal concentration

The results are summarized in Table 2. Among four agents, only two essential oils showed fungicidal effect under 1% with MFC ranging from 0.25% to 0.5%. MFC against *M. furfur* was same in lavender oil and rosemary oil showing 0.5%. Citron seed oil and citrus junos fruit extract had no fungicidal effect on *M. furfur* and *M. sympodialis* under 1%. MFC of lavender oil and rosemary oil was higher against *M. furfur* than that against *M. sympodialis*. For each oil, the MFC value was 2~4 times higher than its respective MIC value.

3. Disc diffusion method

When measuring the diameter of inhibition zone in disc diffusion method, we used agents of 10% except zinc pyrithione (1.56%). The results are shown in Fig. 1 and summarized in Table 3. Citron seed oil and citrus junos fruit extract

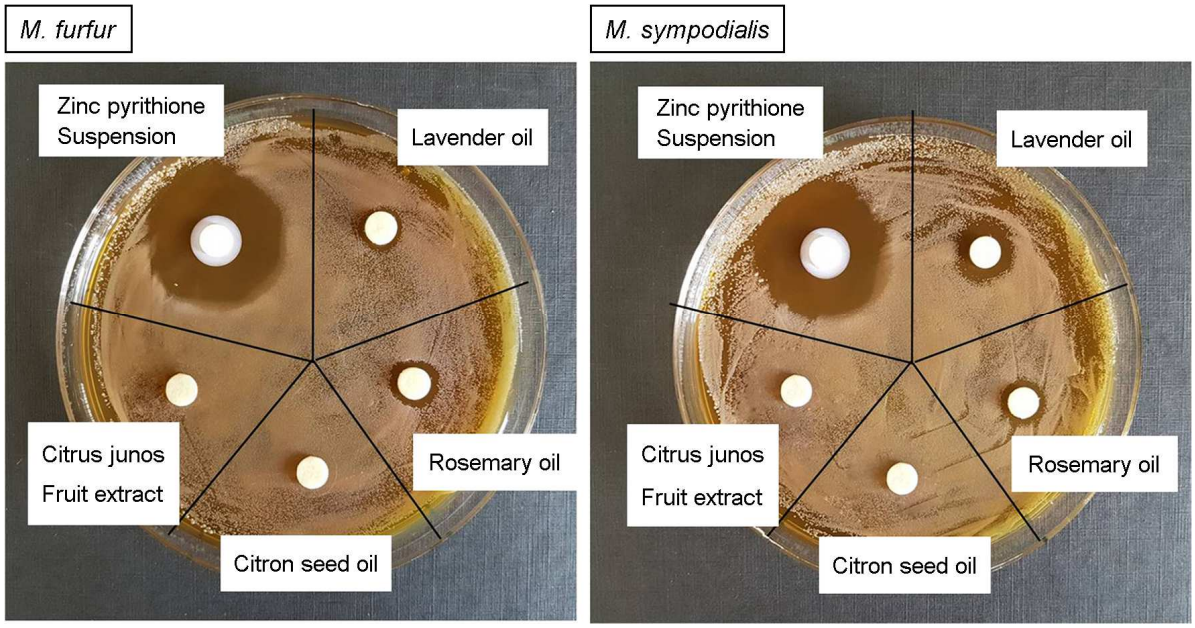

Fig. 1. Inhibitory effects on *Malassezia* species in disc diffusion method

Table 2. The minimal fungicidal concentration (MFC) of natural essential oils and fruit extract

Variables	*M. furfur* (%)	*M. sympodialis* (%)
Lavender oil	0.5	0.25
Rosemary oil	0.5	0.25
Citron seed oil	–	–
Citrus junos fruit extract	–	–
Zinc pyrithione Suspension (control)	0.0025	0.000078

–: no activity under 1%

Table 3. Antifungal activities using disc diffusion method

Variables	*M. furfur* (mm)	*M. sympodialis* (mm)
Lavender oil	9	13
Rosemary oil	9	9
Citron seed oil	–	–
Citrus junos fruit extract	–	–
Zinc pyrithione Suspension (control)	27	26

–: no activity

had no fungicidal effect against *M. furfur* and *M. sympodialis*. Lavender oil inhibited *M. sympodialis* (13 mm) at most; rosemary oil inhibited *M. furfur* and *M. sympodialis* with the same value (9 mm).

4. Clinical trial

Fifteen males and 7 females were enrolled in this study. Mean age was 24.32±2.12. After 2 weeks and 4 weeks of using the shampoo daily, sebum secretion in all 4 area and mean sebum secretion decreased significantly (2 weeks: forehead $p = 0.001$, Rt. temporal area $p = 0.007$, Lt. temporal area $p = 0.001$, vertex $p = 0.004$, mean $p < 0.001$, 4 weeks: forehead $p < 0.001$, Rt. temporal area $p = 0.004$, Lt. temporal area $p < 0.001$, vertex $p = 0.001$, mean $p < 0.001$) (Fig. 2). Erythema on the scalp tended to decrease but it was not statistically significant ($p = 0.157$). Dandruff on scalp also tended to decrease. It was not statistically significant after 2 weeks ($p = 0.157$), but it significantly decreased after 4 weeks ($p = 0.014$). Extent of skin lesion had no improvement after 2 weeks, but it significantly decreased after 4 weeks (Table 4). Clinical photograph and trichoscopic examination (Fig. 3A, 3B) revealed gross improvement of the dandruff and erythema. Adverse

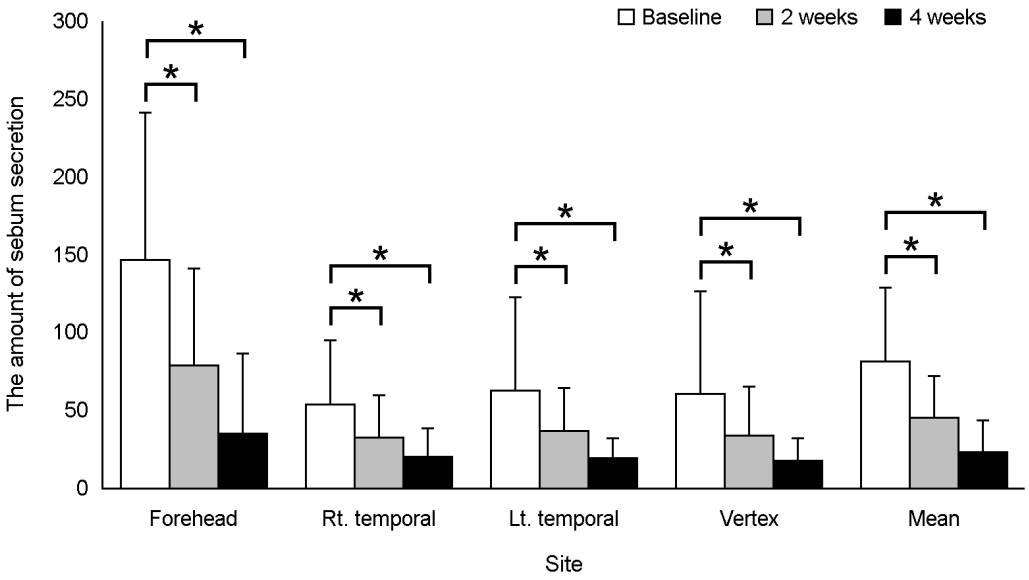

Fig. 2. Sebum secretion of the scalp. Baseline and 4 weeks after treatment (∗p-value < 0.05)

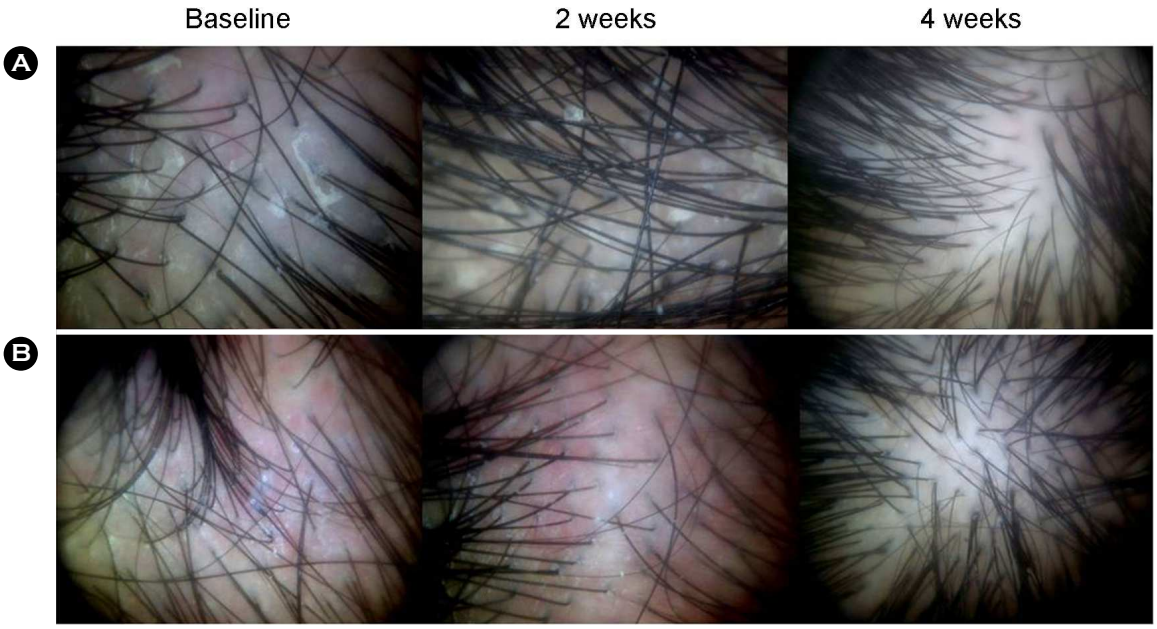

Fig. 3. Trichoscopic examination in two representative subjects: (A) subject 1, (B) subject 2 Erythema and dandruff gradually improved after 2 weeks and 4 weeks compared to baseline.

gross improvement of the dandruff and erythema. Adverse event such as pruritus, swelling, bulla, post-inflammatory hyperpigmentation, or burning sensation was not found.

DISCUSSION

Since fungal colonization is implicated in seborrheic dermatitis and dandruff, antifungal agents are widely used for treatment. Seborrheic dermatitis and dandruff wax and wane

Table 4. The difference between baseline and follow-up scores of erythema, dandruff, and lesion extent

Component	Baseline	2 weeks	4 weeks
Erythema	1.18±0.59	1.09±0.53	1.09±0.53
Dandruff	1.23±0.53	1.14±0.56	0.96±0.38*
Lesion extent	1.68±0.48	1.68±0.48	1.27±0.46*

Values are presented as mean ± standard deviation
*: Significantly different from baseline value (p-value < 0.05), Wilcoxon signed-rank test was used to calculate p-value

chronically; patient compliance is important for disease control. But long-term antifungal treatment may cause problems such as side effects, drug resistance and poor patient compliance [25,26]. A safer and more effective alternative therapy is needed. Many studies are conducted to find new antifungal agents for treatment of dandruff. Naturally derived various plant extracts or essential oils such as bamboo essential oil, *Chamaecyparis obtusa* essential oil and extract, or tea tree oil are known for their effect on the clinical improvement of dandruff[26-28].

In this *in vitro* study, lavender oil and rosemary oil showed relatively low MIC_{50} and MFC among the four agents against *M. furfur* and *M. sympodialis*. MIC_{50} of lavender oil and rosemary oil was lower against *M. sympodialis* when compared with *M. furfur*. Also, MFC of two oils were lower against *M. sympodialis* when compared with *M. furfur*. These showed that different susceptibility against different *Malassezia* species. Only few comparable data are available to validate our study. Lee et al.[23] analyzed the antifungal activities of 108 plant essential oils on *M. furfur*. Lavender oil and rosemary oil were included in that study, but no inhibition zone was detected using disc diffusion method at a concentration of 2 mg/mL. In another study[29], MIC of lavender oil was 4%. The composition and concentration of compound may differ between oils and it depends on plant growth, extraction method, and bioclimatic conditions. Zinc pyrithione, widely used as shampoo for seborrheic dermatitis, showed antifungal effect at very low concentration indicating that this agent was effective. All three experiments assessing antifungal effect (MIC, MFC, disc diffusion method) showed same conclusion that natural essential oils, especially lavender oil and rosemary oil, have antifungal effect on *Malassezia* species. This experiment had some limitations. In previous report, *M. restricta* and *M. globosa* were the dominant species on the scalp with seborrheic dermatitis[30,31]. Only two *Malassezia* species were used in this study, not including *M. restricta* and *M. globosa*. Further experiment with other species may be needed. To apply other *Malassezia* associated diseases, clinical trial may also be also needed.

Therapeutic study has been carried out contemporarily with shampoo containing all four agents. Sebum secretion at all measured area and mean value decreased statistically significantly after 4 weeks. Dandruff, erythema, and lesion extent also significantly decreased after 4 weeks. No adverse event which may affect the patient's compliance was reported. Free fatty acid from natural extract, especially linoleic acid is known for its anti-sebum effect[32]. Citron fruits contain linoleic acid, and we think this might have led to the reduction in sebum secretion[33]. Antifungal effect of rosemary oil and lavender oil seen *in vitro* and anti-sebum effect of citrus fruit seems to have led good clinical outcome after using the shampoo. This synergistic effect of naturally derived oils or extracts shows enough possibility for controlling seborrheic dermatitis and dandruff. In conclusion, this clinical trial shows that shampoo containing natural essential oils and fruit extract is safe and effective for reducing sebum secretion, dandruff, and lesion extent. The therapeutic study has some limitations. Because four extracts were included in the shampoo, whether some individual combined extracts resulted in the clinical outcome is not clear. Through *in vitro* study and clinical trial results, these essential oils and fruit extract may be potentially useful agents for relieving the symptoms of seborrheic dermatitis and dandruff even though further clinical evaluation with a large sample size is needed.

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CONFLICT OF INTEREST

In relation to this article, We declare that there is no conflict of interest.

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