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ORIGINAL ARTICLE

In Vitro Anti-*Malassezia* Activity of *Castanea crenata* Shell and Oil-Soluble *Glycyrrhiza* Extracts

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Background: A new shampoo with anti-*Malassezia* properties obtained from various plants is required to provide seborrheic dermatitis patients with a wider range of treatment options. **Objective:** The aim of this study was to obtain *in vitro* susceptibility profiles of *Malassezia restricta* and *M. globosa*, the most important pathogenic organisms in the development of seborrheic dermatitis, to the plant extracts used in commercial anti-dandruff shampoos. **Methods:** Minimal inhibitory concentrations (MICs) were determined for eight candidate plant extracts and two plant-derived natural products diluted with Leeming and Notman medium to final concentrations of 0.016 to 1 mg/ml. **Results:** *Castanea crenata* shell, *Camellia sinensis* leaf, and oil-soluble *Glycyrrhiza* extracts presented relatively low MIC values (≤0.5 mg/ml) against both strains. The *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts demonstrated especially high anti-*Malassezia* activity, suggesting their potential use in the treatment of seborrheic dermatitis. The extracts also showed fungistatic activity against other common facultative pathogenic yeasts, *Cryptococcus* and *Candida*. **Conclusion:** *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts could potentially be used as active ingredients in anti-seborrheic and anti-dandruff shampoo formulations. They could be helpful for repeated treatments and regular prophylaxis of scalp seborrheic dermatitis. **(Ann Dermatol 29(3) 321～326, 2017)**

-Keywords-
Castanea crenata shell extract, *Malassezia*, Oil-soluble *Glycyrrhiza* extract, Seborrheic dermatitis, Shampoo

INTRODUCTION

Seborrheic dermatitis (SD), including its minor form, dandruff, is an important and common abnormal skin condition affecting about 5% to 10% of the population[1,2]. It is characterized by flaking and scaling of the scalp, accompanied by itch and irritancy. The pathogenesis of SD is not completely understood yet, but a strong association with skin colonization by *Malassezia* yeasts has been suggested[3]. Yeasts of the genus *Malassezia* are members of the human skin flora and are found in 75% to 98% of healthy adults[4]. Most *Malassezia* yeasts are lipid-dependent, requiring an external source of lipids. For this reason, these cutaneous fungi prevail in body areas rich in sebaceous glands, such as the scalp, face, and upper trunk[5]. However, with some predisposing factors, these yeasts can be etiological agents of several skin disorders and uncommon systemic infections. They have been considered opportunistic pathogens of SD, and other conditions, including pityriasis versicolor, atopic dermatitis, folliculitis, and seborrheic blepharitis[6]. With the development of molecular methods, such as polymerase chain reaction, *Malassezia* taxonomy has undergone a great transformation, with the expansion of the genus to the 14 species known today. Among the 14 species, *Malassezia restricta* and *M. globosa* are recognized

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as the most important pathogenic organisms in the development of dandruff and SD. However, an association of *M. sympodialis*, *M. slooffiae*, *Malassezia obtusa*, and *M. furfur* with SD has also been reported in some studies[4].

Owing to its chronic course with remissions and relapses, SD management presents a challenge to clinicians. The condition requires repeated treatment and regular prophylaxis. Medicated and some commercial shampoos containing synthetic antifungal agents in various formulations are frequently prescribed to patients with scalp SD. In particular, ketoconazole and zinc pyrithione shampoos are commonly prescribed[7,8], but do not always have a good outcome. Recently, new shampoos containing natural ingredients, especially various plant extracts with anti-*Malassezia* activity, have been expected to provide patients with a wider range of treatment options.

A number of natural ingredients, especially extracts from various plants with known anti-inflammatory or antimicrobial activities, have been included in commercial shampoos for SD and dandruff. Some ingredients show better than expected effects on SD patients and are presumed to actually have anti-*Malassezia* activity. However, the activities of these natural ingredients against *Malassezia* infections have not been reliably identified yet based on appropriate laboratory determinations through *in vitro* susceptibility testing. In the present study, we evaluated the *in vitro* susceptibility of *M. restricta* and *M. globosa*, the major *Malassezia* species associated with SD, to eight plant extracts, as well as dipotassium glycyrrhizate and phytosphingosine, used in commercial and medicated dandruff shampoos.

MATERIALS AND METHODS

Preparation of reagents

The following eight candidate extracts and two plant-derived natural products were investigated: 3% *Castanea crenata* shell extract, 0.1% *Camellia sinensis* leaf extract, 10% *Artemisia vulgaris* extract, 1% oil-soluble *Glycyrrhiza* extract, 51% *Astragalus membranaceus* root extract, 64.1% *Pinus sylvestris* leaf extract, 75% *Prunus mume* fruit extract, 93.5% *Coix lacryma-jobi* var. *ma-yuen* seed extract, 100% dipotassium glycyrrhizate, and 100% phytosphingosine. All these are ingredients were added to anti-dandruff shampoos based on their anti-inflammatory effects and antimicrobial activities. They were provided by Amorepacific Corporation (Seoul, Korea) as manufactured products.

Organisms

Two isolates, *M. restricta* CBS 7877 and *M. globosa* CBS 7966, previously deposited in a culture collection were used in initial experiments. These yeasts were isolated from clinical samples obtained from human patients with SD. The strains were grown in Leeming and Notman (LN) medium[9]. In addition, *M. sympodialis* KCTC 27514 and *M. slooffiae* KCTC 27517 were used to confirm the antifungal activity of the *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts.

For further evaluation of the antifungal activity of the *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts, we conducted additional experiments with *Cryptococcus neoformans* H99, *Candida albicans* SC5314, *C. tropicalis* KCTC 7512, and *C. parapsilosis* KCTC 7514.

In vitro susceptibility tests

Minimal inhibitory concentrations (MICs) were determined using the method suggested by Sugita et al.[10] with some modifications. Briefly, the extracts/reagents were diluted with 980 μl melted LN agar (LNA) medium to final concentrations ranging from 0.016 to 1 mg/ml. *Malassezia* species were grown in each well and incubated for three days at 34°C to determine MICs.

We further compared the antifungal activity of the *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts to that of ketoconazole. *C. crenata* shell extract, oil-soluble *Glycyrrhiza* extract, and ketoconazole were diluted with 980 μl melted LNA medium to final concentrations ranging from 15.63 to 1,000 μg/ml and 0.015 to 7.68 μg/ml, respectively. *Malassezia* strains were grown in each well of a 24-well plate and incubated for three days at 34°C to determine MICs.

MIC values for *C. neoformans* and the *Candida* strains were determined in 96-well plates using a broth serial dilution method in accordance with the Clinical and Laboratory Standards Institute guideline. Fluconazole was used as a reference standard, and the final concentrations of the test reagents and fluconazole ranged from 0.1 to 1,000 μg/ml and 0.125 to 256 μg/ml, respectively.

RESULTS

The optimal growth of the *Malassezia* species was obtained in LN medium, and the results presented were obtained from four independent experiments. Overall, similar MIC values were obtained in each independently performed *in vitro* susceptibility test.

The MIC values of the eight candidate extracts and two natural products against *M. restricta* and *M. globosa* are presented in Table 1. The *Malassezia* species showed susceptibility to five of the eight candidate extracts, i.e., the *C. crenata* shell, *C. sinensis* leaf, *A. vulgaris*, oil-soluble

Glycyrrhiza, and *A. membranaceus* root extracts. Overall, *M. restricta* was more susceptible to most extracts than *M. globosa*.

The *C. crenata* shell, *C. sinensis* leaf, and oil-soluble *Glycyrrhiza* extracts had relatively low MIC values (≤0.5 mg/ml) against both strains. The *C. crenata* shell extract showed the highest antifungal activity against *M. restricta* among the eight candidate extracts, with the MIC values of 0.065 to 0.125 mg/ml. This was followed by the *A. vulgaris*, oil-soluble *Glycyrrhiza*, *A. membranaceus* root (MICs: 0.125 mg/ml), and *C. sinensis* leaf (MICs: 0.25 mg/ml) extracts. In comparison, for *M. globosa*, the MIC values of the oil-soluble *Glycyrrhiza* extract (0.25～0.5 mg/ml) were lower than those of the other extracts. The *C. crenata* shell and *C. sinensis* leaf extracts followed, with MIC values of 0.5 mg/ml.

Both strains showed *in vitro* resistance to the *P. sylvestris* leaf extract, *P. mume* fruit extract, *Coix lacryma-jobi* var. *ma-yuen* seed extract, dipotassium glycyrrhizate, and phytosphingosine. The MIC values of the *A. vulgaris* and *A. membranaceus* root extracts showed apparent differences in antifungal susceptibility between the two *Malassezia* species.

In the follow-up experiments, the *C. crenata* shell extract, which demonstrated the overall lowest MIC values against *M. restricta* (1st rank of 10 candidates) and *M. globosa* (2nd rank of 10 candidates), showed no activity against the other two *Malassezia* species, *M. sympodialis* and *M. slooffiae*. The oil-soluble *Glycyrrhiza* extract, the other candidate showing anti-*Malassezia* activity against *M. restricta* (2nd rank of 10 candidates) and *M. globosa* (1st rank of 10 candidates), also presented anti-*Malassezia* activity against *M. sympodialis* (MICs: 500 μg/ml) and *M. slooffiae* (MICs: 1,000 μg/ml). Ketoconazole showed activity against all tested *Malassezia* species, with lower MIC values ranging from 0.03 to 0.125 μg/ml (Table 2).

In the experiments with the other yeast organisms, *C. neoformans*, *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, the *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts also demonstrated antifungal activity. The MIC values were considerably lower than those obtained against the *Malassezia* species and ranged from 3.9 to 15.6 μg/ml with the *C. crenata* shell extract and from 6.25 to 50 μg/ml with

Table 1. Minimal inhibitory concentrations values (mg/ml) of 10 candidate extracts and natural products tested against *Malassezia globosa* and *M. restricta*

Candidate extract	*M. restricta* (CBS 7877)	*M. globosa* (CBS 7966)
Castanea crenata shell extract	0.065～0.125	0.5
Camellia sinensis leaf extract	0.25	0.5
Artemisia vulgaris extract	0.125	N/D
Oil-soluble *Glycyrrhiza* extract	0.125	0.25～0.5
Astragalus membranaceus root extract	0.125	N/D
Pinus sylvestris leaf extract	N/D	N/D
Prunus mume fruit extract	N/D	N/D
Coix lacryma-jobi var.*ma-yuen* seed extract	N/D	N/D
Dipotassium glycyrrhizate	N/D	N/D
Phytosphingosine	N/D	N/D

N/D: antifungal activity was not detected.

Table 2. Minimal inhibitory concentrations values (μg/ml) of *Castanea crenata* shell extract, oil-soluble *Glycyrrhiza* extract, and ketoconazole against *Malassezia* species

	M. restricta (CBS 7877)	*M. globosa* (CBS 7966)	*M. sympodialis* (KCTC 27514)	*M. slooffiae* (KCTC 27517)
C. crenata shell extract	62.5～125	500～1,000	N/D	N/D
Oil-soluble *Glycyrrhiza* extract	62.5～125	250～500	500	1,000
Ketoconazole	0.03	0.06～0.125	0.03～0.06	0.125

N/D: antifungal activity was not detected.

Table 3. Minimal inhibitory concentrations values (μg/ml) of *Castanea crenata* shell extract, oil-soluble *Glycyrrhiza* extract, and fluconazole against *Cryptococcus* and *Candida* species

	Cryptococcus neoformans (H99)	*Candida albicans* (SC5314)	*C. tropicalis* (KCTC 7512)	*C. parapsilosis* (KCTC 7514)
C. crenata shell extract	3.9～7.8	7.8	7.8	7.8～15.6
Oil-soluble *Glycyrrhiza* extract	6.25	50	25～50	50
Fluconazole	4～8	2～4	2～4	2

the oil-soluble *Glycyrrhiza* extract. Fluconazole also showed activity (MICs: 2～8 μg/ml) against the *Cryptococcus* and three *Candia* strains (Table 3).

DISCUSSION

Oral and topical antifungal agents became widely used for treatment of dandruff and scalp SD following the confirmation of the etiological relationship mentioned previously[11,12]. In several previous studies, the severity of the disease was positively correlated with the number of microorganisms and lesions, which were generally confined to areas of heavy colonization. During treatment with topical antifungal agents, the yeast load was reduced, and clinical improvement was observed. Furthermore, when the disease recurred, the yeasts were presented in greater numbers[13,14].

In the present study, overall, three candidate extracts, including the *C. crenata* shell, *C. sinensis* leaf, and oil-soluble *Glycyrrhiza* extracts, generated relatively low MIC values against both *M. restricta* and *M. globosa*. This suggests that the extracts have anti-*Malassezia* activities against the major *Malassezia* species associated with SD and may potentially be active components in shampoos for SD. It is interesting that the three active extracts demonstrated much higher antifungal activities than phytosphingosine, which has been commonly used in various cosmetic products because of its anti-inflammatory and antimicrobial potency. It has previously been reported that the growth inhibition of *M. furfur* could be achieved *in vitro* at extremely high phytosphingosine concentrations (mean MIC of 6,250 μg/ml), whereas *C. albicans* strains were inhibited at concentrations between 152 and 269 μg/ml[15].

However, the results showed some differences among the extracts in their effects on the *Malassezia* species. The greatest anti-*Malassezia* activities were detected in the *C. crenata* shell extract against *M. restricta* and in the oil-soluble *Glycyrrhiza* extract against *M. globosa*. The results also showed apparent differences between the two *Malassezia* species in their susceptibility to the *A. vulgaris* and *A. membranaceus* root extracts. This indicates that different *Malassezia* species can have different susceptibilities, which implies the importance of *in vitro* susceptibility tests. Interestingly, the *C. crenata* shell extract, which demonstrated prominent activity against *M. restricta* and *M. globosa*, showed no activity against *M. sympodialis* and *M. slooffiae*. This suggests that the therapeutic activity of the *C. crenata* shell extract against major pathogens of scalp SD could be selective. As previously reported by Kim et al.[16], *M. restricta* and *M. globosa* are predominant *Malassezia* species associated with scalp SD in East Asia, with a frequency of 60% to 100% for each species. In contrast, *M. sympodialis* was found in 11.1% of East Asian scalp SD patients, and *M. slooffiae* was not detected in the above study. The oil-soluble *Glycyrrhiza* extract presented the same or higher activity than the *C. crenata* shell extract against *M. restricta* and *M. globosa* in the follow-up experiment. This suggests that the oil-soluble *Glycyrrhiza* extract is compatible with the *C. crenata* shell extract as an active reagent of anti-SD formulations. The *C. crenata* shell extract and oil-soluble *Glycyrrhiza* extract also showed fungistatic activity against the other common facultative pathogenic yeasts, *Cryptococcus* and *Candida*. The results indicate the potential use of the extracts for other fungal infections. Both ketoconazole and fluconazole, commonly used antifungal agents, also showed positive inhibition, demonstrating that the assays were effective.

Although various medicated and commercial shampoos with ketoconazole and zinc pyrithione have been on the market, patients have been repulsed by long-term exposure to these antifungal medications. As drug-resistant fungal infections have been on the rise over the past decade, plant sources increasingly attract more attention to provide new and more effective antifungal products[17-20]. Corticosteroids used to relieve scalp inflammation seem to be inappropriate for long-term and prophylactic use because of the chronic and relapsing nature of SD. Although steroids show efficacy and safety in short-term treatments, they could lead to frequent relapses shortly after the treatment discontinues[21,22]. Shampoos containing plant extracts with anti-*Malassezia* activity would be an important part in a long-term treatment as an alternative and also for supportive management. In addition, it would be adequate to use them for prophylactic management in patients with mild dandruff or as a maintenance treatment to control SD. If the antifungal effects of the plant extracts are verified, their use would be able to improve the compliance of patients with an aversion to long-term antifungal treatment and steroid use. Compliance is one of the most important factors in the treatment of chronic conditions such as SD and dandruff.

However, this study has the following limitations: the first limitation is related to the interpretation of the results. We do not have sufficient information to evaluate the quality of the study. No reference MIC data for the studied plant extracts against *Malassezia* species have been reported and accumulated before. The second limitation is that we need clinical correlation studies.

Further studies are required to support our results. Above all, it is necessary to identify the actual active compounds in the extracts that are responsible for the antifungal activity. For example, in a previous study that investigated

in vitro anti-*Malassezia* activity of *Asparagus racemosus*, Onlom et al.[23] found through additional experiments that the high saponin content was associated with the antifungal activity. Analyses of interactions between the plant extracts, especially their active compounds, and several vehicles used in shampoo formulations should be performed from as many angles as possible. Vehicles may increase or decrease the activities of antifungal reagents depending on their solubility or chemical interactions. By extension, it would also be meaningful to explore synergistic or antagonistic interactions between the extracts and existing medications, such as ketoconazole and zinc pyrithione. Then, studies that can prove clinical correlations should be conducted to apply these results to clinical fields. The initial severity and characteristics of SD in individual patients should be evaluated, and clinical changes induced by a new formulation should be observed for a certain period. Potential complications and subjective satisfaction, which are important factors for patient compliance, should also be investigated in a series of clinical studies.

Despite the limitations of being a simple observational study with a relatively small sample size, this study is significant in that it reveals the potential of plant extracts, including the *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts, to become active ingredients in anti-SD and -dandruff formulations. Few studies have been conducted to prove the anti-*Malassezia* activity of natural ingredients in shampoos by *in vitro* laboratory tests.

C. crenata is a species of chestnut. The inner shell of chestnut has been used topically as an anti-wrinkle and skin-firming agent for a long time in East Asia. It has also been reported that *C. crenata* shell extract has anti-inflammatory/anti-allergic activity, which inhibits mast cell degranulation[24]. However, no information on its antifungal activity has been previously published to our knowledge. The oil-soluble *Glycyrrhiza* (licorice) extract is a well-known herbal ingredient in various cosmetics for its skin whitening effects. Some reports have described the *in vitro* antimicrobial and antifungal activity of the oil-soluble *Glycyrrhiza* extract. Rohinishree and Negi[25] reported inhibitory efficacy of licorice extract in controlling growth and pathogenicity of *Staphylococcus aureus*. Sato et al.[26] described antifungal activity of the oil-soluble *Glycyrrhiza* extract against *Arthrinium sacchari* and *Chaetomium funicola* and raised the possibility of adding the preparation made from the extract to beverages in polyethylene terephthalate bottles. However, as the dermatologic field lacks scientific evidence for fungistatic activity of the oil-soluble *Glycyrrhiza* extract, little is known on its potential use for the treatment of skin disease. Especially, no research has been reported on its activity against *Malassezia* species associated with SD to our knowledge.

In conclusion, commercial or medicated shampoos containing *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts could be a long-term treatment of choice for SD, although more advanced studies are required. The anti-inflammatory/-allergic activity of the extracts might also help to control symptoms of scalp SD. This study provides the foundation for further research to develop new anti-*Malassezia* shampoos with *C. crenata* shell and oil-soluble *Glycyrrhiza* extracts.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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