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Inhibitory Effects of Seaweed Extracts on Growth of *Malassezia furfur* and *Malassezia restricta*

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Fifty seven species of common seaweed from the coast of Korea were screened for antifungal activity against *Malassezia* species. Seaweeds as a source of bioactive compounds are able to produce a great variety of secondary metabolites with different activities. There are numerous reports on the biological activities of seaweeds against human pathogens, fungi, and yeasts, but only few contain data regarding inhibitory effects against *Malassezia* sp., a major cause of dandruff and seborrheic dermatitis. To help address this paucity of information, this work was carried out to examine the antifungal effects of seaweed extracts against *M. furfur* and *M. restricta*. Of the fifty seven species of marine algae screened for their potential antifungal activity, only 17 species (29.8%) exhibited inhibitory activity. In agar disc diffusion method, the ether extracts of *Corallina pilulifera*, *Enteromorpha linza*, *Laminaria japonica*, *Symphyocladia latiuscula* and *Ulva* sp. showed strong antifungal activity. To identify major constituents in seaweed extracts, four selected extracts were analyzed on a GC-MS equipped with a flame ionization detector, and compared to spectral data from databases WILEY229.LIB and NIST107.LIB. Most constituents in seaweed extracts are fatty acid-related compounds. When we evaluated any acute toxicity, the ether extracts of the selected four species were not toxic in mice. According to these results, it can be suggested that these seaweed extracts are valuable for the development of therapeutic agents in treating dandruff and seborrheic dermatitis. Further investigations to determine its bioactive compound(s) are currently in progress.

Key words: Antifungal activity, Dandruff, *Malassezia* sp., Seaweed extract, Seborrheic dermatitis

Introduction

Malassezia fungi have been the suspected cause of dandruff for more than a century. Previously referred to as *Pityrosporum ovale*, *P. orbiculare*, or *Malassezia*, these fungi are now known to consist of eleven *Malassezia* species, which included ten lipid-dependent species; namely *globosa*, *restricta*, *furfur*, *slooffiae*, *sympodialis*, *japonica*, *nana*, *dermatis*, *yamatoensis* and the non-lipid-dependent *pachydermmatis* (Sugita et al., 2005). Among them, Gemmer et al. (2002) identified *Malassezia* species responsible for dandruff as *M. restricta* and *M. globosa*. These dermatophytes develop microinflammation, parakeratosis and scaling around the hair follicle opening, which are representative symptoms of dandruff (Piérard-Franchimont et al., 2000), a frequently itchy condition. Several fungicidal and fungistatic compounds have been shown to improve dandruff conditions. In particular, against *malassezia* species, several studies demonstrated in vitro activities of antifungals such as azole agents ketoconazole, itraconazole, and fluconazole (Gupta et al., 2000; Sugita et al., 2005), and other compounds such as terbinafine (Leeming et al., 1997) and zinc pyrithione (Piérard et al., 1997).

However, these antibiotics have been known to induce side-effects. Even with the newer azole drugs such as fluconazole, an alarming number of resistant strains are being isolated, while reports of clinical failures and the emergence of resistant strains among patients receiving other azole agents therapies have been increasing (Pfaller and Diekema, 2004; White et al., 1998). Several reports also suggested that in the case of terbinafine and zinc pyrithione, there are side-

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effects such epidermal necrolysis, pustulosis, pigmentation and allergic contact dermatitis if they are used excessively or for extended periods (Cetkovská and Pizinger, 2006; Jo et al., 2005; Nielsen and Menné, 1997).

Interest in marine organisms as potential and promising sources of pharmaceutical agents has increased in recent years (Mayer and Hamann, 2002; Newman et al., 2003). Seaweeds as a source of bioactive compounds are able to produce a great variety of secondary metabolites with different activities. Compounds with antiviral, anthelmintic, antifungal, and antibacterial activities have been detected in green, brown, and red algae (Newman et al., 2003; del Val et al., 2001). There are numerous reports on the biological activities of macroalgae against human pathogens, fungi and yeasts, but only few contain data on their effects against *Malassezia* sp. a major cause of dandruff and seborrheic dermatitis.

The aim of this study was to examine the antifungal effects of seaweed extracts against *M. furfur* and *M. restricta*, while attempting to develop natural therapeutic agents for dandruff and seborrheic dermatitis that have no side effects and high antifungal activity.

Materials and Methods

Seaweed material

These seaweed thalli were collected from the coast of Korea, from November 2005 to April 2006. Fifty seven seaweed species (10 of Chlorophyta, 29 of Phaeophyta and 18 of Rhodophyta) were used in this study. Seaweed tissues were dried for 1 day at room temperature using an electric fan and then ground to powder using a coffee grinder for 10 min. The powder was stored at -20°C until use.

Seaweed extracts

For methanol and water extractions, to each 20 g powder, 1 L of methanol was added for 1 day to extract the methanol-soluble components. This was repeated three times, and the combined extracts were evaporated to dryness. One liter of distilled water was then added to the remaining powder to extract the water-soluble components. For ether extraction, the same procedure was conducted as described for the above methanol extraction. Stock solutions were prepared by the addition of 1 mL of methanol, distilled water or ether to each 100 mg of dried extract. The stock solutions were filtered through a 0.22 μM filter and were stored at -20°C until use.

Microorganisms and media

The fungal strains used in this study were *M. furfur* (KCTC 7846) and *M. restricta* (KCTC 7848). These strains were obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). Stock cultures were maintained on Leeming and Notman medium (1% peptone, 0.5% glucose, 0.01% yeast extract, 0.4% desiccated ox bile, 0.1% glycerol, 0.05% glycerol monostearate, 0.05% Tween 60, 1% high-fat cow's milk, and 1.5% agar in distilled water) at 30°C for *M. furfur* or 34°C for *M. restricta* (Crespo et al., 2000). All strains were grown aerobically in Leeming and Notman agar for disc diffusion assay.

Agar disc diffusion method

At first, in vitro antifungal activity was determined against *M. furfur*. The agar disc diffusion method was followed for antifungal susceptibility test. Spread plates were prepared with the proper concentration of the inocula (1 to 2.0×10^8 CFU/mL). A weighted aliquot of the crude extract dissolved in ether was transferred to 8 mm filter paper (Advantec Filter Paper, Toyo Roshi Kaisha Ltd, Japan) disc and after drying was placed on the center of the seeded agar plate. After 72 hrs of incubation the inhibition zone from the edge of the disc to the inner margin of the surrounding bacterial growth was measured in mm and recorded. Seaweed extracts that showed strong activity were further investigated to confirm the dose response of antifungal activity against *M. furfur and M. restricta*. Controls were also run simultaneously. The antifungal agent ketoconazol (Sigma Co., K-1003) and zinc pyrithione (Sigma Co., H-6377) were included in the assays as positive controls. The experiments were repeated at least three times for each independent assay.

Acute toxicity test

In order to confirm safety of the selected 4 seaweed extracts for the development of therapeutic agents, BALB/c mice (8-10 weeks old; 20-25 g body weight) were used for acute toxicity test (Cho et al., 2007). The animals were kept at room temperate (24±1°C) on a 12 hr light/dark cycle with free access to food and water. For the acute toxicity test, mice were fasted for 6hrs, with water provided *ad libitum*. The seaweed ether extracts were evaporated under vacuum at 35°C using a rotary evaporator and then extracts (5 g/10 mL of 5% Tween 80/kg bw) were administered orally to mice (n=5). The animals were then observed for any abnormal behavior for 3hrs, and mortality was noted for up to 2 weeks. A group of animals treated with Tween-80 served as the control.

Animal experiments were performed in accordance with the U.S. NIH Guidelines for the Care and Use of Laboratory Animals.

GC-MS analysis

To identify major constituents in seaweed extracts, the extracts were analyzed on a GC-MS-QP5050A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, and compared to spectral data from databases WILEY229.LIB and NIST107.LIB.

Results and Discussion

Screening of antifungal activity

Of the fifty seven seaweed species screened for their potential antifungal (=anti-*Malassezia furfur*) activity, none of the methanol and aqueous extracts exhibited such activity. From the ether extracts, only 17 species (29.8%) showed antifungal activity (Table 1). Chlorophyta (green algae) showed the highest activity (50.0%) among the three classes of seaweeds screened. Five Chlorophyta species (*Enteromorpha compressa*, *E. linza*, *Monostroma nitidum*, *Ulva pertusa*, and *Ulva* sp.) showed anti-fungal activity. Among the 29 species of Phaeophyta (brown algae) screened, only five (*Dictyota dichotoma*, *Laminaria japonica*, *Myelophycus simplex*, *Petalonia fascia* and *Sargassum micracanthum*) (17.2%) inhibited fungal pathogens. From the Rhodophyta (red algae) species screened, seven (*Chondaria crassicaulis*, *Chondracanthus tenella*, *Chondrus ocellatus*, *Corallina pilulifera*, *Gracilaria verrucosa*, *Grateloupia elliptica* and *Symphyocladia latiuscula*) (38.9%) showed antifungal activity. The strongest activity among Chlorophyta seaweed species were exhibited by, *E. linza* and *Ulva* sp., among Phaeophyta, *L. japonica* was the highest, while from the Rhodophyta, *C. pilulifera* and *S. latiuscula* showed the highest activity.

Whereas ether extract of seaweeds showed strong antifungal activity, alcohol and aqueous extracts did not showed any inhibitory effect on the growth of fungi in our results. In some previous reports, this tendency was also observed (Rao et al., 1986; Khaleafa et al., 1975). Some of the pronounced algal antibiotic agents have been suggested to be unsaturated, saturated and ethyl ester of fatty acids (Khaleafa et al., 1975; Ara et al., 2005). Therefore, it appears that the anti-*Malassezia* agents from seaweeds are a kind of fatty acids.

From the seaweeds examined, *E. linza*, *Ulva* sp. *L. japonica, C. pilulifera* and *S. latiuscula* showed potent antifungal activity, for which the yield of ether extracts were 0.67, 0.77, 0.78, 0.18 and 0.98%, respectively.

E. linza, *Ulva* sp. and *L. japonica* are commonly used as foodstuff in Korea and Japan (Oh et al., 1990; Okazaki, 1971). Until now, it has not been reported that *C. pilulifera* contains toxic compound(s) in our knowledge. However, *S. latiuscula* is known to contain high concentrations of bromophenols (Wang et al., 2005). It has been reported that some of the bromophenols are toxic to bacteria and other living organisms (Hétu et al., 1983; Calza et al., 2008). Based on this reason, we selected four species (except *S. latiuscula*) for the next assay.

In vitro antifungal activity

The antifungal activity of the selected four seaweed extracts was determined against *M. furfur* and *M. restricta* at 1, 3 and 5 mg/disc concentration, respectively. These results are shown in Table 2. When antifungal activity against *M. furfur* was tested, at 5 mg/disc, the *E. linza* extract showed highest activity, followed by *L. japonica*, *Ulva* sp., and *C. pilulifera*. At 1 mg/disc, the inhibition zone of *L. japonica* ether extract was 2.3 mm, exhibiting the highest activity. The *L. japonica* extract showed highest activity against *M. restricta*, followed by *E. linza and Ulva* sp. at 5 mg/disc. The *C. pilulifera* extract also showed the lowest activity against *M. restricta.* As positive controls, when antifungal activity against *M. furfur* was tested, at 25 μg/disc, the inhibition zones of ketoconazol and zinc pyrithione were 10 and 12 mm, respectively.

Acute toxicity

We evaluated any acute toxicity that the ether extracts of the selected four species might exhibit in mice. Over the 2-week observation period, no death occurred in any mice administered a dose of 5 g/kg bw. Mice administered seaweed extract reacted by wandering for a while and returned to normal behavior after 10 min. According to the WHO (1992), a herbal medicine is considered toxic if the LD50 is lower than 5 g/kg body weight. On this basis, these extracts are not toxic because no mortality was observed at 5 g/kg. Our investigation suggests that the extracts can be safely used by humans at moderate doses.

GC-MS analysis

Yield and major constituents in the selected four seaweed ether extracts are shown in Table 3. GC-MS analysis of the ether extract from *Corallina pilulifera* revealed the presence of hexadecane, undecane, cyclo-

Table 1. Inhibitory effect of seaweed extracts on the propagation of *Malassezia furfur*. 5 mg of seaweed extracts from various samples was loaded onto a disk (8 mm in diameter). The inhibition zones were measured, excepting the 8 mm paper disc. Of the fifty seven species of marine algae screened for their potential antifungal activity, only 17 species showed activity (+, <2 mm; ++, 2 to 5 mm; +++, >5 mm)

Scientific name	Collection site	MeOH	Water	Ether
Chlorophyta				
Bryopsis plumosa	Cheongsapo, Busan	-	-	-
Capsosiphon fulvescens	Gokumdo, Wando	-	-	-
Cladophora sakaii	Cheongsapo, Busan	-	-	-
Codium arabicum	Cheongsapo, Busan	-	-	-
Codium fragile	Cheongsapo, Busan	-	-	-
Enteromorpha compressa	Cheongsapo, Busan	-	-	+
Enteromorpha linza	Cheongsapo, Busan	-	-	+++
Monostroma nitidum	Galmoonri, Wando	-	-	++
Ulva pertusa	Cheongsapo, Busan	-	-	++
Ulva sp.	Sinyang, Jeju	-	-	+++
Phaeophyta				
Colpomenia bullosa	Cheongsapo, Busan	-	-	-
Colpomenia sinuosa	Songjeong, Busan	-	-	-
Costaria costata	Songjeong, Busan	-	-	-
Desmarestia viridis	Cheongsapo, Busan	-	-	-
Dictyota dichotoma	Cheongsapo, Busan	-	-	+
Ecklonia cava	Unseong, Jeju	-	-	-
Ecklonia kurome	Bangeori, Pohang	-	-	-
Ecklonia stolonifera	Daebyeon, Busan	-	-	-
Eisenia bicyclis	Sinyang, Jeju	-	-	-
Hizikia fusiformis	Cheongsapo, Busan	-	-	-
Ishige okamurae	Sachon, Namhae	-	-	-
Ishige sinicola	Cheongsapo, Busan	-	-	-
Laminaria japonica	Cheongsapo, Busan	-	-	+++
Myagropsis myagroides	Cheongsapo, Busan	-	-	-
Myelophycus simplex	Sinyang, Jeju	-	-	++
Pachydictyon coriaceum	Iho, Jeju	-	-	-
Padina crassa	Oro, Jeju	-	-	+
Petalonia binghamiae	Iho, Jeju	-	-	-
Petalonia fascia	Cheongsapo, Busan	-	-	-
Sargassum confusum	Songjung, Busan	-	-	-
Sargassum coreanum	Cheongsapo, Busan	-	-	-
Sargassum horneri	Cheongsapo, Busan	-	-	-
Sargassum micracanthum	Cheongsapo, Busan	-	-	+
Sargassum patens	Cheongsapo, Busan	-	-	-
Sargassum sagamianum	Cheongsapo, Busan	-	-	-
Sargassum thunbergii	Cheongsapo, Busan	-	-	-
Sargassum sp.	Iho, Jeju	-	-	-
Scytsiphon lomentaria	Cheongsapo, Busan	-	-	-
Undaria pinnatifida	Cheongsapo, Busan	-	-	-
Rhodophyta				
Acrosorium polyneurum	Cheongsapo, Busan	-	-	-
Ahnfeltiopsis flabelliformis	Cheongsapo, Busan	-	-	-
Carpopeltis affinis	Iho, Jeju	-	-	-
Chondaria crassicaulis	Cheongsapo, Busan	-	-	++
Chondracanthus tenella	Cheongsapo, Busan	-	-	++
Chondrus ocellatus	Cheongsapo, Busan	-	-	+
Corallina pilulifera	Cheongsapo, Busan	-	-	+++
Gracilaria verrucosa	Cheongsapo, Busan	-	-	++
Grateloupia elliptica	Cheongsapo, Busan	-	-	+
Grateloupia filicina	Iho, Jeju	-	-	-
Grateloupia lunceolata	Cheongsapo, Busan	-	-	-
Helminthocaldia yendoana	Cheongsapo, Busan	-	-	-
Hypnea charoides	Cheongsapo, Busan	-	-	-
Lomentaria catenata	Cheongsapo, Busan	-	-	-
Meristotheca papulosa	Cheongsapo, Busan	-	-	-
Porphyra yezoensis	Cheongsapo, Busan	-	-	-
Prionitis cornea	Cheongsapo, Busan	-	-	-
Symphyocladia latiuscula	Cheongsapo, Busan	-	-	+++

Table 2. Antifungal activity of selected the four species seaweed ether extracts against *Malassezia furfur and M. restricta.* After 72hrs of incubation, the inhibition zone was measured in mm. Data are the averages of triplicate experiment. Statistical significance was calculated using Student's *t*-test and deemed statistically significant at $P<0.01$

Seaweed	*Malassezia furfur*			*Malassezia restricta*		
	1 mg/disk	3 mg/disk	5 mg/disk	1 mg/disk	3 mg/disk	5 mg/disk
Corallina pilulifera	0.0 ± 0.0	1.3 ± 0.5	4.0 ± 1.7	0.0 ± 0.0	1.3 ± 1.5	2.9 ± 1.5
Enteromorpha linza	1.7 ± 0.7	4.0 ± 1.5	8.0 ± 2.0	1.0 ± 0.5	3.0 ± 2.1	5.0 ± 2.7
Laminaria japonica	2.3 ± 0.4	5.0 ± 1.0	7.5 ± 2.1	1.5 ± 0.4	5.7 ± 2.5	8.5 ± 2.7
Ulva sp.	0.7 ± 0.2	2.5 ± 10	6.3 ± 1.8	1.2 ± 0.7	3.0 ± 2.7	5.0 ± 2.6

Table 3. Yield and major constituents of ether extracts from the selected four species

Scientific name	Yield (%)	Major constituents[a]
Corallina pilulifera	0.18	hexadecane, undecane, cyclohexane, and 2-methyl-5,7-dimethylene-1,8-nonadiene
Enteromorpha linza	0.67	8-heptadecene, 9-octadecanoic acid, and 9,12,15-octadecatrien-1-ol
Laminaria japonica	0.78	tridecanoic acid, tetradecanoic acid, pentadecanoic acid, octadecanoic acid, and cyclododecane
Ulva sp.	0.77	heptadecanol, tridecanoic acid, e-3,5-di-tert-butyl-4'-methylstilbene, and 1,2-bis (3,4-dibromocyclohexyl)-1,2-dibromomethane

[a]Major constituents were identified by comparisons of retention times, molecular weights and fragmentation patterns in the databases WILEY229.LIB and NIST107.LIB.

hexane, and 2-methyl-5,7-dimethylene-1,8-nonadiene as major constituents. GC-MS analysis of the ether extract from *Enteromorpha linza* revealed the presence of 8-heptadecene, 9-octadecanoic acid, and 9,12,15-octadecatrien-1-ol as major constituents. GC-MS analysis of the ether extract from *Laminaria japonica* revealed the presence of tridecanoic acid, tetradecanoic acid, pentadecanoic acid, octadecanoic acid, and cyclododecane as major constituents. GC-MS analysis of the ether extract from *Ulva* sp. revealed the presence of heptadecanol, tridecanoic acid, e-3,5-di-tert-butyl-4'-methylstilbene, and 1,2-bis (3,4-dibromocyclohexyl)-1,2-dibromomethane as major constituents.

Marine organisms are increasingly being studied for the screening of biologically active compounds. Among them, seaweeds are considered to be very attractive sources, due to their huge diversity, and relative safety given the fact that, they have long been used in traditional foods and herbal medicines in Asia.

Although several reports have suggested that crude seaweed extracts have antifungal activity (Pesando, 1990), they have primarily focused on antifungal activity against *Trichophyton* spp., and *Candida albicans* (Padmakumar and Ayyakkannu, 1997; Tariq, 1991; Moreau et al., 1984). However, there is a paucity of data on the effects of seaweed extracts against *Malassezia* sp. a major cause of dandruff and seborrheic dermatitis.

The present investigation demonstrated that crude ether extracts of the selected four seaweed species have anti-*Malassezia* activity, without any serious toxic effect at moderate doses. According to these results, it is suggested that these seaweed extracts are valuable for the development of therapeutic agents in dandruff and seborrheic dermatitis. Bioassay-guided fractionation is currently being undertaken to isolate the active compound(s) of species that showed high inhibitory activity during screening.

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