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Chemical Composition and Antifungal Activity of Plant Essential Oils against *Malassezia furfur*

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Malassezia furfur is an important causal factor for seborrheic dermatitis. Nowadays, the drugs available to treat this fungal infection are few. Several studies have documented the biological activity of essential oils. However, its antifungal properties are not completely understood, especially its anti-*Malassezia* activity. The aim of this study were to evaluate the effect of the plant essential oils on the growth of *M. furfur* using disk diffusion method and analyze by Gas chromatography-mass spectrometry (GC-MS) most active essential oils. In first screening, the 17 plant essential oils have possesses inhibitory activity against *M. furfur* at 2 mg/mL. Among the plant essential oils, oil of *Citrus auranifoli* was most active against *M. furfur* and its activity showed dose dependency. This anti-*malassezial* activity was high than that of itraconazole at 2 mg/mL. Oil of *Citrus auranifolia* also was phytochemically examined by GC-MS analysis, its main constituents were identified as limonene, γ-terpinene and terpinolene. It can be concluded that essential oils of *Citrus auranifolia* may have interesting applications to control fungal-derived diseases.

Key words: *Malassezia furfur,* anti-*Malassezia* activity*, Citrus auranifolia,* essential oil, GC-MS analysis

Introduction

In recent years, the genus *Malassezia* has received considerable attention from dermatologists and clinicians [4]. Among them, *Malassezia furfur* has been associated with a variety of cutaneous disease [10, 5].

Ketoconazole is a representative synthetic antifungal imidazolic drug, which is prescribed for skin infections [8, 7]. The widespread use of antifungal agents may have contributed to a shift in species distribution via the emergence of inherently resistant species as significant pathogens [2]. Consequently, novel, broad-spectrum, nontoxic antifungal compounds, appropriate for empirical use and not prone to selection of resistant organisms, are required.

Medicinal plants are increasingly of interest as antimicrobial and antiviral agents and have been widely used in traditional medicine [6]. Nonetheless, little is known about antifungal effect on the genus *Malassezia*. To our knowledge, studies evaluating antifungal effect of essential oils against *M. furfur* have not been reported.

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In the present study, we have analyzed the antifungal properties of plant essential oils on *M. furfur*. The most active *Citrus aurantifolia* essential oil was phytochemically examined by GC-MS analysis, its main constituents were identified.

Materials and Methods

Chemicals

Itraconazole was purchased from Sigma-Aldrich (St. Louis, MO, USA), dissolved in ethyl alcohol, and stored at -20°C. All other chemicals were a reagent grade.

Plant essential oils

Totally 108 plant essential oils were used for antifungal activity tests, and they were purchased from UNIQ F&F Co., Ltd. (Seoul, Korea). The detail information of these essential oils was shown in Table 1.

Malassezia strain, growth conditions and inoculum preparation

Malassezia furfur (KCCM 12679) was obtained from the Korean Culture Center of Microorganisms (Seodeamun-Gu, Seoul, Korea). This strain was grown on Sabouraud

Table 1. Antifungal activities against *Malassezia furfur* of 108 plant essential oils.

Oil	Family	Species	Part	Antifungal activity (cm)
Garlic	Alliaceae	*Allium sativum* L.	root	1.0
Cananga	Annonaceae	*Cananga odorata* Hook fil. et Thomp.	flower	-*
Ylang ylang	Annonaceae	*Cananga odorata* Hook. f. et Thomson	flower	-*
Anise	Apiaceae	*Pimpinella anisum* L.	fruit	-*
Aniseed	Apiaceae	*Pimpinella anisum* L.	fruit	-*
Caraway seed	Apiaceae	*Carum carvi* L.	seed	1.4
Carrot seed	Apiaceae	*Daucus carota* L.	seed	-*
Celery seed	Apiaceae	*Apium graveolens* L.	seed	-*
Coriander	Apiaceae	*Coriandrum sativum* L.	flower	-*
Coriander herb	Apiaceae	*Coriandrum sativum* L.	leaf	1.2
Fennel	Apiaceae	*Foeniculum vulgare* Mill.	seed	-*
Galbanum	Apiaceae	*Ferula galbaniflua* Boiss.et Buhse	root	-*
Lovage root	Apiaceae	*Levisticum officinale* L. Koch	fruit	-*
Parsley herb	Apiaceae	*Petroselinum crispum (*Mill.*)* Nyman	whole plant	-*
Parsley seed	Apiaceae	*Petroselinum crispum (*Mill.*)* Nyman	seed	-*
Star anise	Apiaceae	*Illicium verum* L.	fruit	-*
Armoise	Asteraceae	*Artemesia vulgaris* L.	whole plant	-*
Chamomile blue	Asteraceae	*Chamomilla recutita* (L.) Rauschert	flower	-*
Chamomile roman	Asteraceae	*Chamaemelum nobil* (L.) All.	flower	-*
Davana	Asteraceae	*Artemisia pallens* Wall. Ex DC	whole plant	-*
Helichrysum	Asteraceae	*Helichrysum angustifolium* DC	flower	-*
Tagette	Asteraceae	*Tagetes minuta* L.	leaf	-*
Estragon	Asteraceae	*Artemisia dracunculus* L.	leaf	-*
Wormwood	Asteraceae	*Artemisia absinthium* L.	flower	-*
Frankincense	Burseraceae	*Boswellia thurifera* Roxburgh	root	-*
Myrrh	Burseraceae	*Commiphora myrrha* var. *molmol* Engl.	stem	-*
Tarragon	Asteraceae	*Artemisia dracunculus* L.	stem	-*
Yarrow	Asteraceae	*Achillea millefolium* L.	flower	-*
Cade	Cupressaceae	*Juniperus oxycedrus* L.	wood	-*
Cedarleaf	Cupressaceae	*Thuja occidentalis* L.	leaf	-*
Cedarwood	Cupressaceae	*Juniperus irginiana* L.	bark	-*
Cedarwood Chinese	Cupressaceae	*Juniperus funebris* Endl.	bark	-*
Cedarwood Texas	Cupressaceae	*Juniperus mexicana* Spring.	bark	-*
Cypress	Cupressaceae	*Cupressus sempervirens* L.	twig	-*
Juniperberry	Cupressaceae	*Juniperus communis* L.	berry	-*
Wintergreen	Ericaceae	*Gaultheria procumbens* L.	leaf	-*
Cascarilla bark	Euphorbiaceae	*Croton eleuteria* Bennett	bark	-*
Balsam peru	Fabaceae	*Myroxylon balsamum* var. *pereirae* Royle	resin	1.2
Basil	Lamiaceae	*Ocimum basilicum* L.	flower	-
Basil sweet	Lamiaceae	*Ocimum basilicum* L.	whole plant	-
Catnip	Lamiaceae	*Nepeta cataria* L.	leaf&flower	1.4
Clary sage	Lamiaceae	*Salvia sclarea* L.	flower	-*
Hyssop	Lamiaceae	*Hyssopus officinalis* L.	leaf	-*
Lavender	Lamiaceae	*Lavaendula officinalis* (Chaiz.)	flower	-*
Lavender 10/42	Lamiaceae	*Lavandula angustifolia* Mill.	flower	-*

Table 1. Continued.

Oil	Family	Species	Part	Antifungal activity (cm)
Marjoram	Lamiaceae	*Thymus mastichina* L.	leaf	-*
Melissa	Lamiaceae	*Melissa officinalis* L.	leaf	-*
Oregano	Lamiaceae	*Origanum vulgare* L.	leaf	1.4
Patchouly	Lamiaceae	*Pogostemon cablin* (Blanco) Benth.	leaf	-
Peppermint	Lamiaceae	*Mentha piperita* L.	flower	-
Pennyroyal	Lamiaceae	*Mentha pulegium* L.	leaf	1.0
Rosemary	Lamiaceae	*Rosmarinus officinalis* L.	flower	-*
Sage	Lamiaceae	*Salvia officinalis* L.	whole plant	-*
Sage Dalmatian	Lamiaceae	*Salvia officinalis* L.	leaf	-*
Sage Spanish	Lamiaceae	*Salvia lavandulaefolia* Vahl.	leaf	-*
Savory	Lamiaceae	*Satureja hortensis* L.	leaf	1.4
Spearmint	Lamiaceae	*Mentha spicata* L.	flower	-
Thyme	Lamiaceae	*Thymus vulgaris* L.	leaf	1.4
Thyme white	Lamiaceae	*Thymus vulgaris* L.	leaf	1.3
Cassia especial	Lauraceae	*Cinnamomum cassia* Bl.	bark	-*
Cinnamon bleached	Lauraceae	*Cinnamomum zeylanicum* Garc. Ex Blume Nees	bark	-*
Cinnamon leaf	Lauraceae	*Cinnamomum zeylanicum* Blume	leaf	1.4
Cinnaom leaf oil terpenes	Lauraceae	*Cinnamomum zeylanicum* Garc. Ex Blume Nees	leaf	-*
Rosewood	Lauraceae	*Aniba roseadora* var. amazonica Ducke	wood	-*
Nutmeg	Myristicaceae	*Myristica fragrans* Houtt.	seed	-*
Eucalyptus	Myrtaceae	*Eucalyptus globulus* Labill.	leaf	-*
Eucalyptus 80/85	Myrtaceae	*Eucalyptus globulus* Labill.	leaf	-*
Lemon eucalyptus	Myrtaceae	*Eucalyptus citriodora* Hook	leaf	-*
Myrtle	Myrtaceae	*Myrtus communis* L.	leaf	-*
Niaouli	Myrtaceae	*Melaleuca viridiflora* Sol. Ex Gaertn.	leaf	-*
Pimento berry	Myrtaceae	*Pimenta dioica* (L.) Merr.	flower	1.4
Tea tree	Myrtaceae	*Melaleuca alternifolia* (Maid. & Bet.) Cheel	leaf	-
Clove bud	Oleaceae	*Eugenia caryophyllata* Thumb.	bud	1.4
Clove leaf	Oleaceae	*Eugenia caryophyllata* Thumb.	leaf	1.4
Jasmin absolute	Oleaceae	*Jasminum grandiflorum* L.	flower	-*
Pine	Pinaceae	*Pinus sylvestris* L.	needle	-*
Pine needle	Pinaceae	*Pinus sylvestris* L.	needle	-*
Black pepper	Piperaceae	*Piper nigrum* L.	fruit	-*
Citronella java	Poaceae	*Cymbopogon nardus* L.	leaf	1.3
Geranium	Poaceae	*Pelargonium graveolens* L.	flower	-
Lemongrass	Poaceae	*Cymbopogon citratus* (DC) Stapf.	whole plant	-
Palmarosa	Poaceae	*Cymbopogon martinii* Stapf.	grass	1.2
Vetiver haiti	Poaceae	*Vetiveria zizanioides* L.	root	-*
Bergamot	Rutaceae	*Citrus bergamia* Risso	peel	-*
Buchu	Rutaceae	*Agathosma crenulata* (L.) Pillans	leaf	-*
Buchu leaf	Rutaceae	*Agathosma betulina* (Berg.) Pillans	leaf	-*
Grapefruit	Rutaceae	*Citrus paradisi* Macfadyen	fruit	-*
Orange	Rutaceae	*Citrus sinensis* (L .) Osbeck	peel	-*
Lemon	Rutaceae	*Citrus limonum* L.	peel	-*
Lemon 10F	Rutaceae	*Citrus limonum* L.	peel	-*
Lime	Rutaceae	*Citrus aurantifolia* Swing.	peel	2.6
Lime dis 5F	Rutaceae	*Citrus aurantifolia* Swing.	peel	-*

Table 1. Continued.

Oil	Family	Species	Part	Antifungal activity (cm)
Mandarine	Rutaceae	*Citrus reticulata* Blanco	peel	-*
Neroli	Rutaceae	*Citrus aurantium* L.	flower	-*
Petitgrain	Rutaceae	*Citrus aurantium* L. subp. amara	leaf	-*
Tangerine	Rutaceae	*Citrus reticulata* Blanco	peel	-*
Sandalwood	Santalaceae	*Santalum album* L.	wood	-*
Valerian	Valerianaceae	*Valeriana officinalis* L.	rhizome	-*
Rose	Rosaceae	*Rosa damascene* Mill.	flower	-*
Chamomile blue	Asteraceae	*Chamomilla recutita* (L.) Rauschert	flower	-*
Estragon	Asteraceae	*Artemisia dracunculus* L.	leaf	-*
Lemongrass	Poaceae	*Cymbopogon citratus* (DC) Stapf.	whole plant	-*
Bay	Myrtaceae	*Pimenta racemosa* (Mill.) J.W.Moore	leaf	1.3
Litsea cubeba	Lauraceae	*Litsea cubeba* L.	fruit	-*
Tamanu	*Clusiaceae*	*Calophyllum inophyllum* L.	fruit	-*
Xanthoxylum	Rutaceae	Zanthoxylum armatum	seed	-*
Eucalyptus	Myrtaceae	Eucalyptus citriodora	leaf	-*
Ginger	Zingiberaceae	*Zingiber officinale* Roscoe	rhizome	-*
Itraconazole				1.5

Samples treated with concentration of 2 mg. '-*' expressed no activity.

Dextrose Broth (SDB) or Sabouraud Dextrose Agar (SDA) (Difco, Sparks, MD, USA) supplemented with 1% (v/v) of pure olive oil (Yakuri Pure Chemicals, Kyoto, Japan), following incubation at 37°C during 2-7 days. *Malassezia* strains were maintained on the same medium described previously, at 4°C, with subcultures being carried out on a monthly basis. The same medium was used in all the experiments. Inoculum suspensions were prepared by the method as described previously [14]. One milliliter of 48 h culture was centrifuged (3000 g at 4°C for 1 min), followed by washing the pellets twice with 1 mL of phosphate buffered saline (PBS). Clusters of *Malassezia* cells were formed upon preparation of inoculum suspensions. The washing of these suspensions with PBS promotes single-cell status and more accurate turbidity measurements.

Antifungal activity

The antifungal activity of the plant essential oils was carried out by minor modification of previous method using 100 μL of suspension containing 5×10^6 CFU/mL of *M. furfur* [1]. The discs (6 mm in diameter) were impregnated with 10 μL of essential oil diluted with 95% ethanol under aseptic conditions and placed on the inoculated agar. Negative controls were prepared using the same solvent that was spread on the agar plates. In first screening, the disks (Whatman, 6 mm in diameter) impregnated with essential oil of 2 mg/mL and were placed on the inoculated agar. The selected oil impregnated with 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL also was placed on the inoculated agar. Itraconazole of four concentrations were used as positive reference standards. The antifungal activity was evaluated by measuring the inhibition-zone diameter observed after 48 h of incubation.

Gas chromatography (GC-FID)

Gas chromatography analysis was performed on the Agilent 6890N equipped with a DB-1MS column (30 m× 0.25 mm i.d., 0.25 um film thickness, J&W Scientific, Folsom, CA). The oven temperature was programmed as: isothermal at 40°C for 1 min, then raised to 250°C at 6°C/min and held at this temperature for 4 min. Helium was used as the carrier gas at the rate of 1.5 mL/min in split mode (50 : 1 ratio). The constituents of the plant essential oil were identified by comparing their GC retention indices (RI). RI of each constitutes of plant essential oil were obtained by co-injection of essential oil and a mixture of aliphatic hydrocarbons (C8-C20; Sigma-Aldrich, St. Louis, USA). RI was calculated using the equation proposed by

van Den Dool and Kratz (1963) [15].

Gas chromatography-mass spectrometry (GC-MS)

The essential oils of *Citrus aurantifoliaa* was analyzed on a gas chromatograph (Agilent 6890N)-mass spectrometer (Agilent 5973N MSD) equipped with a DB-5MS column (30 m×0.25 mm i.d., 0.25 um film thickness, J&W Scientific, Folsom, CA). The oven temperature was programmed as for the previous analysis. Helium was used as the carrier gas at the rate of 1.0 mL/min. Effluent of the GC column was introduced directly into the source of the MS via a transfer line (250°C). Ionization voltage was 70 eV and ion source temperature was 230°C. Scan range was 41-450 amu. Compounds were tentatively identified by comparison of mass spectra of each peak with those of authentic samples in the NIST MS library.

Results and Discussion

Antifungal activity of 108 plant essential oils was found when the essential oil was assayed at 2 mg/mL (Table 1). The results showed the inhibitory effects of 17 oils [*Allium sativum* L., *Carum carvi* L., *Coriandrum sativum* L., *Myroxylon balsamum* var. *pereirae* Royle, *Nepeta cataria* L., *Origanum vulgare* L., *Mentha pulegium* L., *Satureja hortensis* L., *Thymus vulgaris* L. *Thymus vulgaris* L., *Cinnamomum zeylanicum* Blume, *Pimenta dioica* (L.) Merr., *Eugenia caryophyllata* Thumb.(Clove bud), *Eugenia caryophyllata* Thumb. (Clove leaf), *Cymbopogon nardus* L., *Cymbopogon martinii* Stapf., *Citrus aurantifolia* Swing. and *Pimenta racemosa* (Mill.) J.W.Moore] on *M. furfur* at 2 mg/mL. These oils showed similar inhibitory activity to itraconazole at 2 mg/mL. Among the 17 active oils, only the *Citrus auranifolia* essential oil exhibited strong inhibitory activity at dose dependent manner and the activity was higher than itraconazole at same concentration (Fig. 1).

The chemical compositions of the antifungal essential oil are shown in Table 2. A total of 15 compounds were identified in *Citrus auranifolia* oil by GC and GC-MS analysis. Among the identified compounds, limonene (51.07%) was the most abundant compound and γ-terpinene (14.29%) and terpinolene (11.32%) were followed (Table 2).

Malassezia species are associated with a number of dermatological disorders including dandruff/seborrheic dermatitis and pityriasis versicolor [3]. In general, for treatment of such illnesses, azole drugs such as fluconazole and ketoconazole are used, but with increasing usage of antifungal agents, those have led undesired effects include severe toxic hepatitis, acquired cutaneous adherence [12, 13]. In contrast, there are a few reports concerning the susceptibility of *Malassezia* to natural antifungal or anti-*Malassezia* agents. Consequently, plant-derived anti-fungal agents are of increasingly interest for the development of new, more effective and specific anti-*Malassezia* agents

In this work, we demonstrated that the 17 oils among the 108 plant essential oils had inhibitory activity against *M. furfur* at 2 mg/mL. The most active *C. aurantifolia* essential oil among the 17 oils was some higher than that of itraconazole showing dose dependency (Fig. 1).

Hence, the *C. auranifolia* essential oil has most good antifungal activity among tested oils. This activity of *C. auranifolia* essential oil can be associated with the presence of limonene, γ-terpinene, and terpinolene. However, also other major and/or minor components in the *C. auranifolia* essential oil may influence its antifungal activity. Possible synergistic and/or antagonistic interactions among the essential oil components should also be considered. There have been studies supporting these results in various *Citrus* species and its components [9, 11, 16].

In conclusion, the essential oils of *C. auranifolia* showed interesting antifungal activity. Although, further studies are

Table 2. Chemical composition of *Citrus aurantifolia* essential oil.

Compound	RI *	Relative composition ratio, %
α-pinene	928	0.58
β-pinene	967	1.12
Myrcene	981	0.91
3-Carene	1,002	1.58
αTerpinene	1,007	3.02
p-Cymene	1,010	2.71
Limonene	1,019	51.07
Unknown 1	1,038	0.50
γ-Terpinene	1,049	14.29
Terpinolene	1,078	11.32
Unknown 2	1,125	0.47
α-Terpineol	1,171	7.44
Benzoic acid	1,179	1.38
β-Caryophyllene	1414	0.76
α-Cedrene*	1,432	1.12
α-Bergamotene*	1,496	1.32
α-Bisabolene†	1,529	0.43

* Retention indices.
† Tentatively identified by mass library.

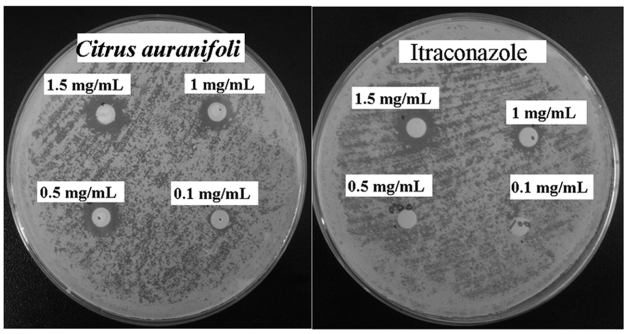

Fig. 1. Antifungal activity of essential oil of *Citrus auranifolia* against *Malassezia furfur.* Values represent the means of 3 independent experiments.

needed, the use of essential oils of *C. auranifolia* against microbial growth seems a valuable alternative as antifungal compound, especially in the cases of anti-*Malassezia* resistance.

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국문초록

비듬균(*Malassezia furfur*)에 대한 식물 오일들의 항균활성 및 활성오일의 성분 분석

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비듬균(*Malassezia furfur*) 는 두피 질환을 일으키는 중요한 요소로써 오늘날 이러한 균들을 치료하는 치료제는 거의 없다. 몇몇의 전의 연구에서 오일들의 다양한 생물학적 활성이 보고되어졌지만 그것들의 항비듬균 활성은 거의 연구되지 않았고, 특히 *Malassezia furfur*에 대한 저해 활성은 보고되어 있지 않다. 따라서, 이 연구에서는 *Malassezia furfur*에 대한 식물 오일들의 저해활성을 평가하고, 이러한 오일 중에서 가장 활성이 좋은 오일을 가스 크로마토그래피(Gas chromatography-mass spectrometry)에 의해 성분을 분석하였다. 1차적으로 108개 오일의 스크리닝 과정에서 17개 오일[*Allium sativum* L., *Carum carvi* L., *Coriandrum sativum* L., *Myroxylon balsamum* var. *pereirae* Royle, *Nepeta cataria* L., *Origanum vulgare* L., *Mentha pulegium* L., *Satureja hortensis* L., *Thymus vulgaris* L. *Thymus vulgaris* L., *Cinnamomum zeylanicum* Blume, *Pimenta dioica*(L.) Merr., *Eugenia caryophyllata* Thumb.(Clove bud), *Eugenia caryophyllata* Thumb.(Clove leaf), *Cymbopogon nardus* L., *Cymbopogon martinii* Stapf., *Citrus aurantifolia* Swing. and *Pimenta racemosa*(Mill.) J.W.Moore] 이 2 mg/mL 의 농도에서 대조구인 이크라코나졸(itraconazole)과 비슷한 활성을 나타내었다. 이러한 17개 오일 중에서 *Citrus auranifolia* 오일이 가장 활성이 좋았으며, 처리농도 (1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL와 0.1 mg/mL) 에 따라 활성이 감소하는 경향을 보였다. 또한 *Citrus auranifoli* 오일의 가스크로마토그래피(GC-MS) 성분분석 결과 limonene, γ-terpinene and terpinolene의 함유율이 높은 것으로 나타났다. 그러므로 *Citrus auranifolia* 오일은 곰팡이 유래 질병들을 치료하기 위한 활성성분을 함유하고 있는 것으로 보인다.