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Original Article

Antioxidant Properties of 7 Domestic Essential Oils and Identification of Physiologically Active Components of Essential Oils against *Candida albicans*[1]

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ABSTRACT

In this study, we selected two essential oils, *Citrus unshiu* and *Cinnamomum cassia* with superior antioxidant effects from the essential oils of 7 wild plants in South Korea and examined their antimicrobial activity against *Candida albicans*, which causes dermatitis to identify the antimicrobial components in the essential oils. As a result of measuring DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity, SC_{50} value of the *Citrus unshiu* essential oil was 0.010 mg/mL, while for the *Cinnamomum cassia* essential oil, SC_{50} value was 0.09 mg/mL. In addition, when ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity was measured, SC_{50} value of the *Citrus unshiu* essential oil was 0.09 mg/mL, while for the *Cinnamomum cassia* essential oil, it was 0.06 mg/mL, exhibiting high antioxidant activity. For the minimum inhibitory concentration (MIC), the essential oil of *Cinnamomum cassia* was 1.25 mg/mL and that of *Citrus unshiu* was 5 mg/mL, demonstrating a high antimicrobial activity of the *Cinnamomum cassia* essential oil. Through the thin layer chromatography (TLC) bioassay, we assessed the antimicrobial activity against *C. albicans* according to the fraction components of the two essential oils. Also, by using preparative TLC (prep. TLC), we obtained the active fractions, and by performing GC/MS analysis of the components with the same R_f value, we identified the antimicrobial-active components. As a result, the main components having antioxidant and antimicrobial activities were cinnamyl acetate, eucalyptol, linalool, and citral of the *Cinnamomum cassia* essential oil and linalool from the *Citrus unshiu* essential oil. Also, based on the analysis of the fractional components that showed antioxidant and antimicrobial activities in both of the two essential oils, it was found that linalool has antioxidant activity, while cinnamyl acetate, eucalyptol, citral, and geranyl acetate have antioxidant and antimicrobial activities.

Keywords: essential oil, cinnamomum cassia, sargentii, citrus unshiu, antioxidant effect, antimicrobial effect

1. INTRODUCTION

Recently, as the respiratory syndrome caused by SARS-CoV-2 infection, known as COVID-19, has become more serious, the World Health Organization (WHO) declared it as a pandemic, the highest level of risk assessment. In addition, the WHO announced that the number of infections caused by COVID-19

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worldwide exceeded 36 million and that the mortality rate also reached 3.5 %. While the development of vaccines against COVID-19 is undergoing, it is difficult to distribute them due to the prolonged clinical trials and there are no preventive measures proposed by center for disease control (CDC) other than practicing personal hygiene, such as washing hands and wearing masks. However, skin diseases such as dermatitis, eczema, and hives, etc. could be caused by wearing a mask for an extended time, and in particular, there is a concern about a skin disorder in humid conditions caused by *Candida albicans*, a pathogenic yeast (Mary *et al*., 1968). Also, due to the scarcity of masks, a secondary infection by other pathogenic microorganisms from re-using the masks is a threat to the public health (Ravine *et al*., 2020).

C. albicans is an opportunistic fungus that resides in human body and it multiplies and causes inflammation when the immunity weakens. Vaginal Candidiasis is a typical disease caused by *C. albicans* infection and symptoms such as watery vaginal discharge, painful urination, dyspareunia, and burning sensation may occur (Park *et al*., 2018). Once *C. albicans* proliferate in the oral mucosa, the oral candidiasis may occur, causing white pseudomembrane to from in the oral cavity and macerated mucosa leads to bleeding (Mayer *et al*., 2013). Also, candidemia is a disease with high prevalence and mortality, which is known to be caused mostly by *C. albicans* (Trick *et al*., 2002).

Meanwhile, it has been reported that COVID-19 infection promotes cell aging by increasing oxidative stress along with diseases such as aging, diabetes, cancer and hypertension (Marie *et al.*, 2020). Oxidative stress induced by viral infection was first published in the Sendai virus infection study in 1979 (Peterhans *et al*., 1979). NADPH-linked dehydrogenase is activated through the binding of the virus with the cells to generate reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide, which induces oxidative stress (Zhang *et al*., 2019). These free radical species generated through this process can cause skin aging and also act as a risk factor for cancers or lifestyle diseases (Sies, 2003).

A number of studies have been conducted on substances with antioxidant and antimicrobial activities. In addition to these studies, synthetic drugs with antioxidant and antimicrobial activities have been continuously developed (Sen *et al.*, 2010). Representative antioxidants include ethoxyquin, propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and other phenolic compounds, which are used as cosmetic preservatives (Chen *et al*.,1992). Representative antimicrobial agents are ketoconazole, itraconazole, and terbinafine, etc. (Mattew *et al.*, 1993). Ketoconazole is used to treat diseases caused by fungi such as ringworm, candidiasis, and seborrheic dermatitis (Min *et al*., 1992). Itraconazole is an antimicrobial used to treat onychomycosis, candidal vaginitis, dermatophytosis, mycotic keratitis, and oral candidiasis and is effective against fungi such as *C. albicans* and *Aspergillus fumigatus* (Denning *et al*., 1997). Terbinafine is mainly used to treat the mycotic infection on nails, but it has been reported that terbinafine also shows antimicrobial activity against *Trichophyton rubrum*, which causes acne (Mukherjee *et al*., 2003).

Despite these effects, however, long-term use of synthetic compounds may cause various side effects, including kidney toxicity, headache, and dermal hypersensitivity (Del *et al.*, 2005). Thus, there has been a demand for the development of antioxidants and antimicrobial agents that are not harmful to the human body with minimized side effects. Accordingly, interest in substances derived from natural resources is increasing (Koehn *et al*., 2005). Among natural substances, plant essential oils have been reported to have diverse composition with physiological activities. A typical nat-

ural product, plant essential oils are volatile organic compounds extracted from flowers, leaves, stems, and roots and are secondary metabolites, which are essential for plants to survive. It is known that such plant essential oils are composed of terpene compounds, mainly monoterpenes, and have antioxidant and antimicrobial effects (Luis *et al*., 2019; Sharma *et al*., 2017).

Recently, interest in cypress tree is increasing, which contains vast amount of phytoncide, a substance exhibiting antimicrobial activity in plant essential oils. The essential oil of cypress shows antioxidative as well as antimicrobial activities against *Listeria monocytogenes, T. rubrum,* etc. and was evaluated to be safe in cytotoxicity tests. Beside the cypress tree, essential oils of eucalyptus, Japanese red pine, and Korean white pine have been reported to have anti-oxidative, anti-inflammatory, and anti-allergic effects (Seo *et al*., 2015). Japanese larch essential oil is also known to have anti-fungal activity against *Pidermophyton floccosum, Trichophyton* mentagrophytes, and T. rubrum, which are dermatophytes (Kim et al., 2013). The commercial value of these plant essential oils with high physiological activity has continuously increased. However there is a limit, in which they do not have antimicrobial activity against all strains, and for South Korea, most of the raw materials of the essential oils are being imported.

Therefore, in this study, we established basic data for the development of natural antioxidants and antimicrobial agents by evaluating antioxidant and antimicrobial activity of 7 domestic plant essential oils that have not been studied in South Korea. We also aimed to identify active components based on domestic plant essential oils, which have both antioxidant and antimicrobial activities.

2. MATERIALS and METHODS

2.1. Test materials

The domestic wild species used in this study are *Cinnamomum loureiroi* Nees, *Juniperus chinensis*, *Zanthoxylum schinifolium* S. et Z., *Zanthoxylum piperitum*, *Artemisia capillaris* Thunb., *Citrus unshiu*, *Citrus pseudogulgul* hort. and the collection areas are shown in Table 1. Leaves, fruits, and fruit shells were stored frozen at −39°C in green condition for hydrodistillation. We evaluated the antioxidant activity on 7 plant essential oils and the antimicrobial activity was evaluated by selecting essential oils showing the antioxidant activity. *C. albicans* (ATCC 10231), which causes skin diseases, was obtained from the Korea Culutre Center of Microorganism, and cultured in a sabouraud dextrose agar (SDA, BD ProbeTec™ ET, Becton−Dickinson Microbiology System, Sparks, MD, USA) medium at 26~28°C for 4 days.

2.2. Extraction of the plant essential oils

Each plant essential oil was extracted by hyro-

Table 1. Information of plants for essential oils

Scientific name	Part	Region	Yields (%, w/w)
Cinnamomum loureirii Nees	Leaves	Jeju Island	0.58
Juniperus chinensis	Leaves	Ulleungdo island	1.69
Zanthoxylum schinifolium S. et Z.	Fruits	Jinju	2.34
Zanthoxylum piperitum	Fruits	Jinju	2.97
Artemisia capillaris Thunb.	Aerial parts	Jeju Island	0.30
Citrus unshiu	Peels	Jeju Island	5.26
Citrus pseudogulgul hort.	Peels	Jeju Island	5.35

distillation. 1 kg of the test plant sample was added to a 10 L round-bottom flask, and distilled water was added to the extent that the sample was immersed. Then, the plant essential oils were collected using a 102±1°C heating mantle (MS-DM608 heating mantle, MTOPS®, Yangju, Korea) and a dean stark trap. Moisture remaining in the essential oils was removed with anhydrous sodium sulfate (Samchun, 98.5%, Korea) and the obtained essential oils were stored in a refrigerator at 4°C.

2.3. Evaluation of the antioxidant activity of the plant essential oils

2.3.1. Measurement of DPPH radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ratio was measured by modifying the methods of Blois (1958) and Sharma (2009). After dissolving DPPH in 95% ethanol (Samchun, Korea) to a concentration of 0.1 M, it was mixed 1:1 with the essential oil. The final concentration of the solution was adjusted to be 3, 2, 1, 0.5, 0.25, and 0.125 mg/mL and reacted in the darkroom for 30 minutes. The absorbance was measured using a UV-Vis spectrophotometer (Shimadzu uv-1601 pc spectrophotometer, kyoto, Japan) at a wavelength of 517 nm. DPPH radical scavenging ratio was calculated using the following equation (1). Also, a calibration curve was plotted in the range of 0-100 μM for the positive controls, trolox, ascorbic acid, and tocopherol. Based on this, the equivalent amount of the plant essential oil to the standard substance was estimated in the unit of SC_{50} value of standard substance (μ mol) versus SC_{50} value of plant essential oil (per 100g).

DPPH radial scavenging ratio (%) =

$$\{1-(As/Ac)\} \times 100 \quad (1)$$

As: Absorbance of the sample
Ac: Absorbance of the control

2.3.2. Measurement of ABTS radical scavenging activity

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was measured by modifying the methods of Re (1999) and Konan (2016). 8 mL of 7 mM ABTS and 2 mL of 12.25 mM aqueous potassium persulfate solution (Samchun, Korea) were mixed and reacted in a darkroom at 37°C for 16 hours. Then, the mixture was diluted with ethanol so that the absorbance at a wavelength of 734 nm was 0.70 (±0.02). The diluted $ABTS^{\bullet+}$ solution and essential oil were mixed at 24:1 ratio, which was adjusted to the final concentration of 2, 1, 0.5, 0.25, and 0.125 mg/mL for the essential oil. Finally, after the mixture was reacted in the darkroom at 37°C for 5 minutes, the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 734nm. ABTS radical scavenging ratio was calculated using the following equation (2). Also, a calibration curve was plotted in the range of 0-100 μM for the positive controls trolox, ascorbic acid, and tocopherol. Based on this, the equivalent amount of the plant essential oil to the standard substance was estimated in the unit of SC_{50} value of standard substance (μ mol) versus SC_{50} value of plant essential oil (per 100g).

ABTS radical scavenging ratio (%) =

$$\{1-(As/Ac)\} \times 100 \quad (2)$$

As: Absorbance of the sample
Ac: Absorbance of the control

2.3.3. TLC-DPPH(ABTS) Antioxidant analysis

On a thin layer chromatography (TLC) plate (TLC silica gel 60 F_{254}, merck, 20 × 20 cm, 0.2 mm thickness) eluted with n-Hexane:Ethyl acetate (8:1) solution, DPPH or ABTS radical solution was sprayed to visualize the group of active substances. Based on the stained TLC plate, the ratio of the distance moved by the active substance group to the distance moved by the eluent (R_f value) was calculated. prep. TLC

(TLC silica gel 60 F_{254}, merck, 20 × 20 cm, 0.2 mm thickness) was conducted using the same eluent and the corresponding substances were extracted using ethyl acetate from silica gel based on the R_f value. For the quantitative and qualitative analysis of the extracted substances, GC/MS component analysis was performed.

2.4. Antimicrobial activity of the selected essential oil

2.4.1. Measurement of the anti-proliferative activity against pathogenic microorganisms

To evaluate the antimicrobial activity of the plant essential oils, the disk diffusion assay was conducted according to the protocol suggested by the Clinical and Laboratory Standards Institute (CLSI, USA). To prepare a solution at a concentration of 1×10^6 CFU/mL (0.5 McFarland turbidity standard), *C. albicans* was diluted with distilled water and adjusted to absorbance of 0.12~0.15 at a wavelength of 530 nm by using a UV-vis spectrometer (Shimadzu, Japan). After inoculating the diluted *C. albicans* inoculum into a Mueller-Hinton agar supplemented with 2% glucose and methylene blue (MH-GMB, 0.5 μ g/ml) medium, a paper disk with a diameter of 8 mm in which 20 μL of 100 and 50 mg/mL essential oil was absorbed, was placed and cultured at 26~28°C for 4 days. The diameter of the clear zone formed around the disk was measured, and the central part of the disk and the boundary of the clear zone were collected to observe the change in the cell morphology of *C. ablicans* using the scanning electron microscope (SEM).

2.4.2. MIC Measurement

To investigate the minimum inhibitory concentration (MIC) of *C. albicans*, microdilution was performed according to the protocol suggested by CLSI. The 1×10^6 CFU/mL *C. albicans* solution (0.5 McFarland turbidity standard) was diluted 1000 times to adjust the concentration to 1×10^3 CFU/mL. 100 μL of YB broth medium was dispensed to a 96-well plate and 100 μL of the essential oil was dispensed for each concentration. Then, 100 μL of the diluted *C. albicans* inoculum was added, followed by incubation at 26~28°C for 4 days to measure the MIC.

2.4.3. Morphology analysis of *C. albicans*

SEM analysis was conducted by modifying the method suggested by Insuan & Chahomchuen (2020). After inoculating *C. albicans* to SDA, an 8 mm paper disk absorbed with 20 μL of 100 mg/mL essential oil was placed and incubated at 26~28°C for 4 days. The central part and the boundary part of the clear zone around the disk were collected and attached to aluminum stub using carbon tape, followed by the treatment with 2% osmium tetroxide vapor for 24 hours to fix *C. albicans*. After coating with gold for 2 minutes using Gold sputter, the surface area change of *C. albicans* was observed at a magnification of 10,000~20,000 using SEM (Supra 55vp, Carl Zeiss, Germany).

2.4.4. TLC Bioassay analysis

The essential oils used in this study are a mixture of various organic substances. Among these substances, TLC bioassay was conducted to detect the major components having antimicrobial activity. Plant essential oils were eluted on a TLC plate using n-Hexane:Ethyl acetate (8:1) as an eluent and observed at UV 254 nm to divide the active fraction. The eluted TLC plate was cut along the elution direction and placed on a solid medium 5 minutes after the *C. albicans* inoculation. Then, it was cultured at 26~28°C for 4 days to identify the clear zone and the fraction groups with the same R_f value as the active fraction were extracted. Finally, GC/MS component analysis was conducted.

2.5. GC/MS analysis

For the GC/MS component analysis in this study, all samples were analyzed according to the following conditions using tridecane as an internal standard. DB-5 column (25 m × 0.32 mm × 0.52 μm) was used for the GC (model-Agilent 7890, USA) analysis. Helium was used as the carrier gas. The injector temperature was 260°C and the detector temperature was 280°C. The oven temperature was maintained at an initial temperature of 50°C for 5 minutes and then increased by 5°C/min to reach the final temperature of 250°C and maintained for 10 minutes. For mass spectrometry, Agilent 5937 was used and it was analyzed in EI mode. The structure of a compound was identified by comparing the mass data of the obtained sample peak with the standard library data (Willy 7th ed).

3. RESULTS and DISCUSSION

3.1. Antioxidant activity of the essential oil

3.1.1. DPPH radical scavenging activity

The DPPH radical scavenging activity was measured for *C. loureirii* Nees, *J. chinensis*, *Z. schinifolium* S. et Z., *Z. piperitum*, *A. capillaris* Thunb., *C. unshiu*, and *C. pseudogulgul* hort. to investigate the antioxidant activity of the plant essential oils and the results are shown in Fig. 1. The DPPH radical scavenging activity is an antioxidant index for phenolic substances such as flavonoids and phenolic acids and it is known that the higher the reducing power, the higher the radical scavenging activity (Kang *et al*., 1995). Among 7 plant essential oils, the DPPH radical scavenging activities of the essential oils of *C. unshiu* and *C. loureirii* at a concentration of 3 mg/mL were 95% and 94%, respectively, showing superior antioxidant activity compared to 24% for *C. pseudogulgul* essential oil, 91% for *A. capillaris*, 23% for *Z. piperitum*, 83% for *J. chinensis*, and 60% for *Z. schinifolium*.

Using trolox (water-soluble, vitamin E analog), ascorbic acid (vitamin C), and tocopherol (vitamin E), which are currently used as antioxidants, as positive controls, SC_{50} value, the concentration when the DPPH radical scavenging activity reaches 50%, and the equivalent compared to the standard were calculated. Table. 2 shows the SC_{50} values of the positive controls and the sample plant essential oils. The SC_{50} values of each plant essential oils were as follows: 0.82 mg/mL for *A. capillaris*, 1.25 mg/mL for *J. chinensis*, 1.98 mg/mL for *Z. schinifolium*, 7.41 mg/mL for *C. pseudogulgul*, 8.42 mg/mL for *Z. piperitum*, 0.10 mg/mL for *C. unshiu*, and 0.09 mg/mL for *C. loureirii*. That is, the SC_{50} values of the *C. unshiu* and *C. loureirii*

Table 2. SC_{50} value determined in the DPPH assay of essential oils and equivalence value compared to reference compounds

	SC_{50} (mg/mL)	Equivalence value		
		Trolox	Ascorbic acid	Tocopherol
C. unshiu	0.1	4.8	3.28	0.37
C. loureirii Nees	0.09	5.35	3.65	0.42
A. capillaris Thunb.	0.82	0.57	0.39	0.04
J. chinensis	1.25	0.37	0.26	0.03
Z. schinifolium S. et Z.	1.98	0.23	0.16	0.02
C. pseudogulgul hort.	7.41	n/a	n/a	n/a
Z. piperitum	8.42	n/a	n/a	n/a

n/a : not applicable
Equivalence value = SC_{50} value of reference compound (μmol) / SC_{50} value of essential oil (100 g)

essential oils were lower than those of other essential oils. Also, when comparing the equivalent compared to the standard substance, the *C. unshiu essential oil had an equivalent of* trolox 4.80, ascorbic acid 3.28, and tocopherol 0.3, while the *C. loureirii* essential oil had an equivalent of trolox 5.35, ascorbic acid 3.65, and tocopherol 0.42. In other words, the equivalent of the *C. unshiu* and *C. loureirii* essential oils were higher than that of other 5 plant essential oils. Based on these results, it is thought that the essential oils of *C. unshiu* and *C. loureirii* have high antioxidant activity. This is a similar result to the previous studies (Kim, 2013; Yang *et al*., 2012) in which the maximum DPPH radical scavenging activity of *C. unshiu* was 95% and the SC_{50} value of the *C. loureirii* essential oil was 0.073 mg/mL.

3.1.2. ABTS radical scavenging activity

In addition to the DPPH radical scavenging activity, ABTS radical scavenging activity was measured to investigate the antioxidant activity of 7 plant essential oils and the results are shown in Fig. 2. Among 7 plant essential oils, the ABTS radical scavenging activities of the essential oils of *C. unshiu*, *C. loureirii*, and *J. chinensis* at a concentration of 3 mg/mL were 95%, 96%, and 97%, respectively, showing superior antioxidant activity compared to 80% for *C. pseudogulgul*, 37% for *A. capillaris*, 78% for *Z. piperitum*, and 91% for *Z. schinifolium*.

For each plant essential oil, the SC_{50} values and the equivalent to the positive controls trolox, ascorbic acid, and tocopherol were calculated, and the results are shown in Table. 3. The SC_{50} values of the plant essential oils were as follows: 0.08 mg/mL for *J. chinensis*, 0.25 mg/mL for *Z. schinifolium*, 0.10 mg/mL

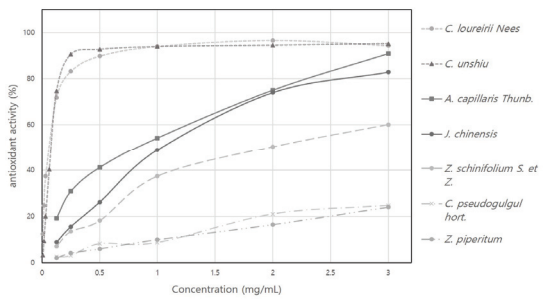

Fig. 1. DPPH radical scavenging ability of essential oils.

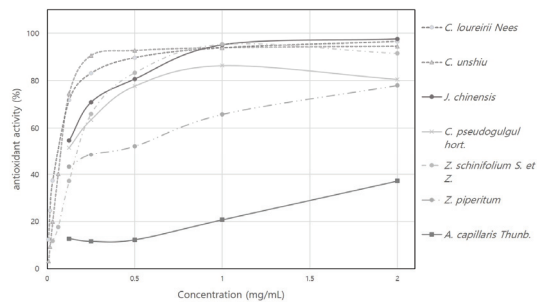

Fig. 2. ABTS radical scavenging ability of essential oils.

Table 3. SC_{50} value determined in the ABTS assay of essential oils and equivalence value compared to reference compounds

	SC_{50} (mg/mL)	Equivalence value		
		Trolox	Ascorbic acid	Tocopherol
C. unshiu	0.09	2.69	2.05	3.92
C. loureirii Nees	0.06	3.94	3.01	5.75
A. capillaris Thunb.	4.05	n/a	n/a	n/a
J. chinensis	0.08	4.33	2.26	2.97
Z. schinifolium S. et Z.	0.25	1.33	0.7	0.91
C. pseudogulgul hort.	0.1	3.33	1.74	2.29
Z. piperitum	0.29	1.16	0.61	0.8

n/a : not applicable
Equivalence value = SC_{50} value of reference compound (μmol) / SC_{50} value of essential oil (100 g)

for *C. pseudogulgul*, 0.29 mg/mL for *Z. piperitum*, 0.09 mg/mL for *C. unshiu*, 0.06 mg/mL for *C. loureirii*, and 4.05 mg/mL for *A. capillaris*. That is, the SC_{50} values of *C. unshiu* and *C. loureirii* essential oils were relatively lower than those of other essential oils. Also, the equivalent of *J. chinensis* essential oil to the standard substance was trolox 4.33, ascorbic acid 2.26, and tocopherol 2.97, while that of *C. unshiu* essential oils was trolox 2.69, ascorbic acid 2.05, and tocopherol 3.92, and that of *C. loureirii* essential oil was trolox 3.94, ascorbic acid 3.01, and tocopherol 5.75. In other words, the equivalent of these three essential oils was relatively higher than that of the other essential oils. Based on these results, it is suggested that the essential oils of *J. chinensis*, *C. unshiu*, and *C. loureirii* have a high antioxidant activity.

3.1.3. GC/MS component analysis and TLC analysis

Table 4 shows the component analysis results of *C. loureirii* and *C. unshiu* essential oils. The main components of *C. loureirii* essential oil were linalool (21.27%) and α -citral (8.62%), while those of *C. unshiu* essential oil were limonene (69.87%) and γ -terpinene (6.83%). These results are similar to the previous studies in which linalool was detected in *C. loureirii* essential oil, and limonene and γ -terpinene in *C. unshiu* essential oil (Xiao et al., 2013; Seshadri et al., 2020). The essential oils of *C. loureirii* and *C. unshiu* were eluted on a TLC plate using n-Hexane: Ethyl acetate (8:1) as an eluent. After dividing the fraction as shown in Fig. 1, the R_f values were assigned.

3.1.4. Analysis of antioxidant components in essential oils

Through the evaluation of DPPH radical scavenging activity and ABTS radical scavenging activity, it was found that the essential oils of *C. unshiu* and *C. loureirii* showed the antioxidant activity with a low SC_{50} value. *C. unshiu* essential oil showed the antioxidant activity at *C. unshiu* B and *C. loureirii* essential oil showed the antioxidant activity at *C. loureirii* C, D, and E. Table 5 shows the results of GC/MS component analysis on the fractions with antioxidant activity. Components such as β -myrcene, linalool, and elemol were detected in *C. unshiu* B. Cinnamyl acetate, eucalyptol, linalool, and citral were detected as the main components of *C. loureirii*. In *C. loureirii* C, eucalyptol, α -Citral, and geranyl acetate, while linalool and α -citral in *C. loureirii* D, and linalool, β -citral, α -citral, and cinnamyl acetate in *C. loureirii* E.

Table 4. Major compounds of *C. unshiu* and *C. loureirii* Nees essential oils by GC/MS analysis

C. loureirii Nees essential oil			*C. unshiu* essential oil		
Retention time (min)	Relative proportion (%)	Compounds	Retention time (min)	Relative proportion (%)	Compounds
7.542	2.11	α-Pinene	7.523	0.86	α-Pinene
10.74	3.51	Eucalyptol	8.87	0.49	β-Pinene
13.391	21.27	Linalool	10.64	5.17	β-Myrcene
15.802	1.19	Terpineol	11.131	69.87	Limonene
17.376	6.50	β-Citral	11.803	6.83	γ-Terpinene
17.953	4.03	Geraniol	12.582	0.40	Terpinolen
18.285	8.62	α-Citral	13.099	0.72	Linalool
18.382	1.68	Bornyl acetate	15.394	0.17	Terpinen-4-ol
22.649	3.20	Cinnamyl acetate	15.833	0.37	α-Terpineol

Table 5. Major compounds of the antioxidative fraction by GC/MS analysis

	Retention time (min)	Compounds
C. unshiu A	10.598	β-Myrcene
	13.043	Linalool
	25.038	Elemol
C. loureirii Nees C	10.693	Eucalyptol
	18.297	α-Citral
	21.063	Geranyl acetate
C. loureirii Nees D	13.42	Linalool
	18.164	α-Citral
C. loureirii Nees E	13.183	Linalool
	17.367	β-Citral
	18.207	α-Citral
	22.657	Cinnamyl acetate

3.2. Antimicrobial activity of essential oils

3.2.1. Measurement of the anti-proliferative activity against pathogenic microorganism

It is known that a plant essential oil has antimicrobial activity when the diameter of the clear zone around the 8 mm paper disk increases, which is absorbed with a plant essential oil in the medium inoculated with pathogenic microorganisms. The disk diffusion method was conducted for *C. unshiu* and *C. loureirii* essential oils which exhibited excellent radical scavenging ability among 7 plant essential oils. As shown in Fig. 4(a), a clear zone was formed in *C. loureirii* essential oil at a concentration of 100 mg/mL. However, no clear zone was formed in *C. unshiu* essential oil at a concentration of 100 mg/mL and 50 mg/mL. The diameters of the clear zone of the *C. loureirii* essential oil were 44.9±8.9 mm at a concentration of 100 mg/mL and 33.4±5.5 mm at a concentration of 50 mg/mL. Based on these results, it was confirmed that *C. unshiu* essential oil cannot inhibit the growth of *C. albicans*, but *C. loureirii* essential oil has antimicrobial activity against *C. albicans*.

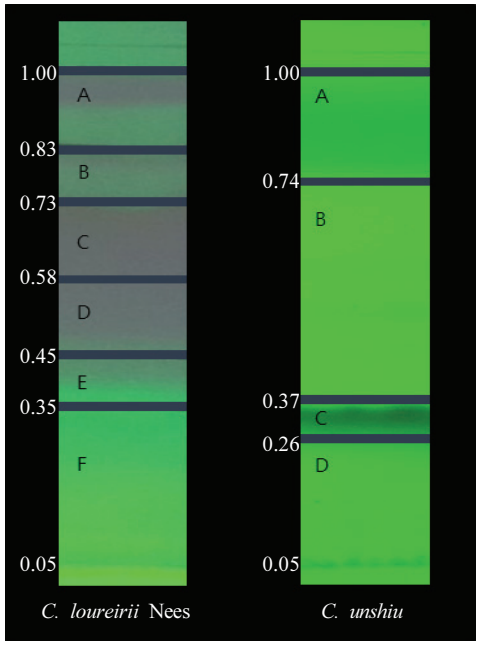

Fig. 3. TLC images and Rf values of *C. loureirii* Nees and C. unshiu essential oils.

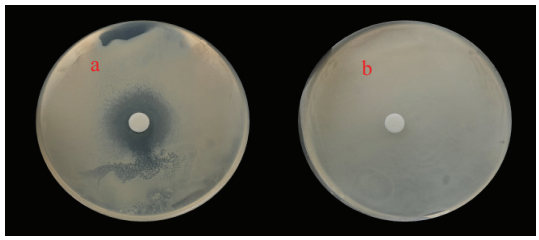

Fig. 4. Zones of inhibition (mm) showing antibacterial activity of (a) *C. loureirii* Nees and (b) *C. unshiu* essential oils.

3.2.2. MIC Measurement

After *C. albicans* was exposed to the plant essential oil, it was incubated at 26~28℃ for 4 days. The turbidity was visually examined, and the minimum bactericidal concentration (MBC) and MIC were measured. For *C. albicans*, the MIC of *C. loureirii* essential oil was measured low as 1.25 mg/mL, while that of *C. unshiu* essential oil was measured relatively high as 5 mg/mL. For the MBC, *C. loureirii* essential oil has

a value of 1.25 mg/mL and *C. unshiu* essential oil has a value of 5 mg/mL. The *C. loureirii* essential oil shows high antimicrobial activity even compared to the essential oils of cypress, Japanese cedar, and Japanese red pine. The essential oils of cypress, Japanese cedar, and Japanese red pine all had a MIC value of 2.18 mg/mL or more against *C. alibicans*. Also, the MIC values of these oil were 2.18 mg/mL or more against Candida genus such as *Candida krusei*, *Candida glabrata*, *Candida tropicalis*, and *Candida pseudotropicalis*, showing lower antimicrobial activity than that of the *C. loureirii* essential oil (Lee *et al*., 2001; Lee *et al*., 2009). It is assumed that the essential oil of *C. loureirii* has a high antimicrobial activity against *C. albicans*, whereas the *C. unshiu* essential oil has a low antimicrobial activity against *C. albicans*.

3.2.3. Morphology changes of the strain

Fig. 5 shows the SEM images to visualize the phenomenon on the strain surface in which plant essential oil inhibits the growth of *C. albicans*. To compare the difference in the degree of growth depending on the diffusion effect of essential oils in the medium, we compared the SEM images by dividing them into the control image of *C. albicans* (Fig. 5(control)), the boundary and central part of the clear zone (Fig. 5(a,b,c,d)). In case of the strain treated with the *C. unshiu* essential oil, the growth of the strain was not inhibited, so the strain of the part where the paper disk contacted was collected and analyzed by SEM. As a result, it was found that the surface was smooth, and growth was not inhibited as shown in the SEM images (Fig. 5(b)). On the other hand, in the case of the part collected after removing the paper disk, the strains were hardly observed, which may be attributed that the contact of the disk prevented the strain growth. In contrast, it was observed that the cell wall of *C. albicans* was destroyed and contracted when it was treated with the *C. loureirii* essential oil which has antimicrobial activity. Such result may be due to the destruction of the cell wall by the essential oil as well as the loss of internal organelles by the difference in concentration between the inside and the outside of the cell. At the boundary of the clear zone, undamaged *C. albicans* and damaged *C. albicans* were observed at the same time. In Fig. 5(c), a SEM image of the inside of the clear zone treated with the *C. loureirii* essential oil, it was observed that the *C. albicans* cells were separated from each other and contracted without connection.

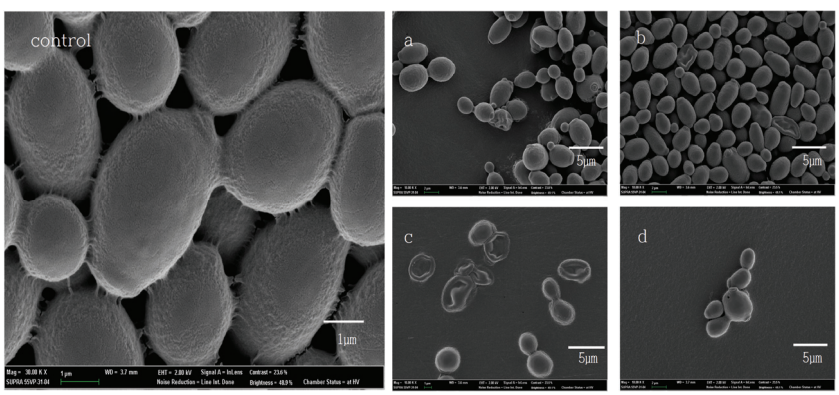

Fig. 5. SEM images of *C. albicans* control and exposed to essential oils; (a) the boundary of and (c) inside the zone of inhibition formed by *C. loureirii* Nees essential oil, (b) the boundary of and (d) inside the zone of inhibition formed by *C. unshiu* essential oil.

3.2.4. TLC bioassay

The clear zone on the TLC plate was observed in the C, D, and E parts of the *C. loureirii* essential oil. The result of the GC/MS analysis on those parts is shown in Table 6. The main components detected in each part were as follows: cinnamyl acetate, eucalyptol, linalool, and citral for the *C. loureirii* essential oil, eucalyptol, α -citral, and geranyl acetate for *C. loureirii* C, linalool, and α -citral for *C. loureirii* D, and linalool, β -citral, α -citral, and cinnamyl acetate for *C. loureirii* E. Eucalyptol in the *C. loureirii* essential oil was reported to have antimicrobial activity against *S. aureus* and citral, which was detected in *C. loureirii* C, D, and E, was also reported to have antimicrobial activity (Karlović *et al*., 2000; Saddiq *et al*., 2010). In the evaluation of the anti-proliferative activity against *C. Albicans*, the diameter of the clear zone treated with citral was 40 mm or more, which is similar to the diameter of the clear zone treated by the *C. loureirii* essential oil in this study. Such result suggests that the *C. loureirii* essential oil has a high antimicrobial activity. In addition, it has shown excellent microbial activity against other Candida strains, and the diameters of the clear zone were 27.5 mm for *Candida parapsilosis*, 19.7 mm for *C. krusei*, and 32.6 mm for *C. tropicalis* (Silva *et al*., 2008). The essential oil of *Cinnamomum verum*, whose main component is cinnamyl acetate, was also reported to have antimicrobial activity against *Candida albicans* with a MIC values of 0.12 mg/mL (Unlu *et al*., 2010; de Lima Carvalho *et al*., 2018). Geranyl acetate was reported to have antimicrobial activity against Candida strains with a MIC value of 20 μl/mL against *C. krusei*, 5 μl/mL against *C. parapsilosis*, and 1.25 μl/mL against *C. guillermondii*. It also has a MIC value of 0.32 μl/mL against *T. rubrum* and *T. mentagrophytes*, showing excellent antimicrobial activity against various strains (Goncalves *et al*., 2012). Eucalyptol, citral, cinnamyl acetate, and geranyl acetate were found to exhibit antimicrobial activity. Linalool, which was detected at the fraction of the essential oil showing antioxidant and antimicrobial activity, was also detected in the *C. unshiu* essential oil, which did not show antimicrobial activity. In the previous study, as the result of the disk diffusion test using linalool on *C. albicans*, the diameter of the clear zone was 12 mm and the MIC value of was 1.23 mg/mL, indicating that linalool has good antimicrobial activity (Herman *et al*., 2016). Also, it showed high antimicrobial activity against the strains other than *C. albicans*; the MIC value of 0.1 mg/mL on *Aggregatibacter actinomycetemcomitans*, 0.8 mg/mL on *Porphylomonas gingivalis*, and 0.2 mg/mL on *Prevotella intermedia* (Hsu *et al*., 2013). Linalool was also detected in the *C. unshiu* essential oil, but it is presumed that the essential oil did not show antimicrobial activity as linalool present amounts with a low content of 0.72%.

Table 6. Major compounds of the antimicrobial fraction by GC/MS analysis

	Retention time (min)	Compounds
C. loureirii Nees C	10.693	Eucalyptol
	18.297	α-Citral
	21.063	Geranyl acetate
C. loureirii Nees D	13.42	Linalool
	18.164	α-Citral
C. loureirii Nees E	13.183	Linalool
	17.367	β-Citral
	18.207	α-Citral
	22.657	Cinnamyl acetate

4. CONCLUSION

In this study, we selected the essential oils of *C. loureirii* and *C. unshiu* that have excellent antioxidant activity by evaluating the antioxidant activity of 7 plant essential oils. We also investigated antimicrobial activity of two selected essential oils against *C.*

albicans. The TLC-DPPH (ABTS) analysis was conducted with the *C. loureirii* and *C. unshiu* essential oils, and their active components were identified by GC/MS analysis. The *C. unshiu* essential oil was mainly composed of linalool, while the *C. loureirii* essential oil had eucalyptol, α -citral, β -citral, and linalool as the main components. As a result of the evaluation of antimicrobial activity for two essential oils, the *C. unshiu* essential oil showed no antimicrobial activity, whereas the *C. loureirii* essential oil exhibited high antimicrobial activity with a MIC value of 1.25 mg/mL. This is also a high value when compared to the previous study that reported the essential oils of lavender (*Lavandula officinalis*), rosemary (*Rosmarinus officinalis*), and eucalyptus (*Eucalyptus globulus*) had a MIC value of 5 mg/mL against *C. albicans* (Tampieri *et al*., 2005). Through the TLC-bioassay, we confirmed that the *C. loureirii* C, D, and E fractions have antimicrobial activity, and eucalyptol, citral, geranyl acetate, and cinnamyl acetate were detected in the active fractions. In conclusion, we suggest that the *C. loureirii* essential oil, which has antioxidant activity and antimicrobial activity against *C. albicans* would be a promising candidate for solving skin problems in the future. Particularly, active components in the *C. loureirii* C, D, and E fractions have potential to be developed as a functional cosmetic with both antioxidant and antimicrobial activities.

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APPENDIX

(Korean Version)

식물정유 7종의 항산화능 분석 및 *Candida albicans* 생장 억제 정유의 생리활성 성분 구명

초록 : 본 연구에서는 국내 자생식물 7종의 정유 중 우수한 항산화 효과가 있는 온주밀감, 육계나무 정유 2종을 선별하였고, 피부염을 유발하는 *Candida albicans*에 대한 항미생물 활성 평가를 통해 식물정유의 항미생물 활성성분을 구명하고자 하였다. 1,1-diphenyl-2-picrylhydrazyl (DPPH) 라디칼 소거활성 측정 결과 SC_{50}값이 온주밀감 정유 0.010 mg/mL, 육계나무 정유 0.09 mg/mL을 나타냈으며, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) 라디칼 소거활성 측정 결과 온주밀감 정유 0.09 mg/mL, 육계나무 정유 0.06 mg/mL로 높은 항산화 활성을 나타냈다. Minimum Inhibitory Concentration는 육계나무 정유 1.25 mg/mL, 온주밀감 정유 5 mg/mL 값으로 육계나무 정유에서 뛰어난 항미생물 활성이 나타났다. Thin layer chromatography (TLC) bioassay를 통해 2종 정유의 성분 분획에 따른 *C. albicans*에 대한 항미생물 활성을 평가하였고, preparative TLC (prep. TLC)를 통해 활성분획을 획득하고, 동일 R_f값을 가지는 성분들에 대해서 GC/MS 분석을 수행하여 항미생물 활성성분을 구명하였다. 그 결과 항산화 및 항미생물 활성을 나타내는 주성분은 각각 육계나무 정유의 경우 cinnamyl acetate, eucalyptol, linalool, citral 온주밀감 정유의 경우 linalool이었다. 2종 정유에 공통적으로 항산화 및 항미생물 활성을 나타낸 분획부 성분분석 결과 linalool은 항산화, cinnamyl acetate, eucalyptol, citral, geranyl acetate은 항산화와 항미생물 능력이 있다고 사료된다.

1. 서 론

최근 COVID-19로 알려진 SARS-CoV-2 감염에 의한 호흡기 증후군이 심각해짐에 따라 세계보건기구 (WHO)에서는 전염병 경보단계 중 최고 위험 등급인 pandemic을 선언하였고, COVID-19로 인한 전 세계 감염자가 3천6백만 명을 넘었으며 치사율 또한 3.5%에 다다른다고 발표하였다. COVID-19에 대한 백신은 아직 현재 개발 중에 있으나 임상시험 단계의 장기화로 인해 보급이 어려운 상황이며, 질병관리청에서 제시한 예방 방법 또한 올바른 손 씻기나 마스크를 착용하는 방법 외에는 예방책이 전무하다. 하지만 이 또한 장시간 마스크 착용으로 인해 피부 염증, 습진, 두드러기 등 피부질환이 유발될 수 있으며 특히 습한 조건에서 유발되는 피부질환균인 *Candida albicans*에 의한 피부병 문제가 염려되고 있다(Mary *et al*., 1968). 또한 마스크 품귀현상으로 인하여 마스크 재사용 및 방치에 따라 *C. albicans*을 포함한 다른 병원성미생물에 의한 2차 오염문제가 국민 보건환경을 위협하고 있다(Ravine *et al*., 2020).

*C. albicans*는 우리 몸에 상주하는 균으로 면역력이 약해지면 균이 증식하여 몸에 염증을 일으킨다. 칸디다 질염은 *C. albicans*의 균에 의해 감염되는 대표적인 질병으로 질 분비물, 배뇨통, 성교통, 작열감 등의 증상을 나타낸다(Park *et al*., 2018). 구강 점막에서 증식이 일어나면 구강 칸디다증에 감염되게 되고 구강 내 흰 가막이 생기며 점막이 짓무르고 출혈이 발생하게 된다(Mayer *et al*., 2013). 또한 칸디다 혈증은 유병율과 치사율이 높은 질환으로 *C. albicans*가 가장 흔한 균주로 알려져 있다(Trick *et al*., 2002).

한편, COVID-19의 감염으로 인해 노화, 당뇨병, 암, 고혈압과 같은 질환과 함께 산화적 스트레스가 가중되어 세포노화가 촉진된다고 보고된 바 있다(Marie *et al*., 2020). 바이러스 감염에 의해 유발된 산화적 스트레스는 1979년 Sendai virus 감염 연구에서 처음 발표되었다(Peterhans *et al*., 1979). 바이러스와 세포의 결합을 통해 NADPH-linked dehydrogenase 효소가 활성화되어 superoxide anion, hydrogen peroxide와 같은 활성산소 즉, reactive oxygen species (ROS)가 형성되며, 이로 인해 산화적 스트레스가 유발된다(Zhang *et al*., 2019). 이러한 과정을 통해 생성된 활성산소는 피부노화의 원인이 되고 암이나 성인병을 촉발시키는 위험 요소로 작용할 수 있다(Sies. 2003).

병원성 미생물에 의한 질환과 활성산소 발생 문제에 대한 해결방안으로서 항산화 및 항미생물 성분에 관한 연구가 다수 이루어지고 있다. 이러한 연구 추세와 더불어 항산화, 항미생물에 효능이 있는 합성의약품이 꾸준히 개발되어 왔다(Sen *et al*., 2010). 대표적인 항산화제로는 ethoxyquin, propylgallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 기타 페놀류 등이 있으며 화장품 보존제 등으로 사용되고 있다(Chen *et al*.,1992). 대표적인 항미생물제로는 ketoconazole, itraconazole, terbinafine 등을 들 수 있다(Mattew *et al*., 1993). Ketoconazole같은 경우 백선, 칸디다증, 지루성 피부염 등에

효과적이며 곰팡이에 의한 치료에 사용된다(Min *et al*., 1992). Itraconazole은 손발톱 진균증, 칸디다성 질염, 피부 사상균증, 진균성 각막염, 구강 칸디다증의 치료에 사용되며 *C. albicans*나 *Aspergillus fumigatus*와 같은 진균류에 효과가 있는 항미생물제이다(Denning *et al*., 1997). Terbinafine은 손톱의 표면 진균 감염을 치료로 위해 주로 사용되지만 여드름을 유발하는 균으로 알려진 *Trichophyton rubrum*에도 항미생물 활성을 나타낸다는 연구도 진행된 바 있다(Mukherjee *et al*., 2003).

하지만 이러한 효능들에도 불구하고 합성 물질을 오랜 기간 사용하게 되면 신장독성유발, 두통 및 피부 과민 반응과 같은 다양한 부작용을 야기할 수 있다(Del *et al.*, 2005). 이러한 부작용을 최소화하여 몸에 해롭지 않은 항산화 및 항미생물제의 개발이 요구되고 있으며, 이에 따라 자연 유래 물질에 대한 관심이 높아지고 있다(Koehn *et al*., 2005). 자연 유래 물질 중 식물정유의 경우 다양한 성분조성을 가지며 생리활성을 나타낸다고 보고된 바 있다. 대표적인 천연물질인 식물정유는 식물의 꽃, 잎, 줄기, 뿌리 등으로부터 추출하여 얻은 휘발성 유기물질로, 식물의 생명유지에 있어서 필수적인 2차 대사산물이다. 정유는 모노테르펜류를 중심으로 하는 테르펜 화합물이 주성분을 이루며 항산화, 항미생물 등에 효능이 있다고 알려져 있다(Luis *et al*., 2019; Sharma *et al*., 2017).

최근 식물정유 내 항미생물 활성을 나타내는 물질인 피톤치드를 많이 함유하고 있는 편백나무에 대한 관심이 높아지고 있다. 편백나무 정유는 항산화 효과 뿐만 아니라 *Listeria monocytogenes, T. rubrum* 등에 대한 항미생물 활성을 나타내며, 세포독성 실험에서도 안전한 것으로 평가됐다. 편백나무 뿐만 아니라 유칼립투스, 소나무, 잣나무 정유에서도 항산화, 항염, 항알러지 효과가 있는 것으로 보고되었다(Seo *et al*., 2015). 일본잎갈나무 정유 또한 피부사상균인 *Pidermophyton floccosum, Trichophyton mentagrophytes, T. rubrum*에 대하여 항진균 활성을 가지는 것으로 알려져 있다(Kim *et al*., 2013). 이러한 뛰어난 생리활성을 지닌 식물정유의 산업적 이용가치는 계속적으로 증대되고 있으나, 정유가 모든 균주에 대해서 항미생물 활성을 가지지 못한다는 한계가 있으며, 국내의 경우 원료 정유는 대부분 수입에 의존하고 있는 실정이다.

따라서 본 연구는 우리나라 미발굴 된 식물정유 7종에 대하여 항산화 활성과 항미생물 활성 평가를 통해 천연 항산화제와 항미생물제에 개발에 기초자료를 구축하고, 항산화와 항미생물 효과를 동시에 가지는 국내 식물정유 기반 유효 성분을 밝히고자 한다.

2. 재료 및 방법

2.1. 공시재료

본 연구에 사용된 국내 자생 수종은 육계나무 (*Cinnamomum loureirii* Nees), 향나무 (*Juniperus chinensis*), 산초나무 (*Zanthoxylum schinifolium* S. et Z.), 초피나무 (*Zanthoxylum piperitum*), 사철쑥 (*Artemisia capillaris* Thunb.), 온주밀감 (*Citrus unshiu*), 사두감 (*Citrus pseudogulgul* hort.)으로 채취 지역은 Table 1과 같다. 수증기 증류법 (hydrodistillation)으로 정유를 추출하기 위해 잎, 열매와 열매껍질은 생재상태로 −39°C에서 냉동 보관하였다. 대상 식물정유 7종에 대한 항산화 평가를 진행하였으며, 항산화 활성을 나타낸 정유를 선별하여 항미생물 활성을 평가하였다. 한국 미생물 보존센터로부터 공시균주인 피부 질병을 유발하는 *C. albicans* (ATCC 10231)을 분양받았으며, sabouraud dextrose agar (SDA, BD ProbeTec™ ET, Becton–Dickinson Microbiology System, Sparks, MD, USA) 배지에 26~28°C에서 4일간 배양하였다.

2.2. 정유의 추출

각 대상 식물정유는 수증기 증류법을 통해 추출하였다. 10 L 둥근 플라스크에 1 kg의 대상 식물 시료를 넣고 시료가 잠길 정도의 증류수를 첨가하여. 102±1°C 가열용 맨틀(MS-DM608 heating mantle, MTOPS®, Yangju, Korea)과 dean stark trap을 이용하여 정유를 수집하였다. 정유에 남아 있는 수분은 anhydrous sodium sulfate (Samchun, 98.5%, Korea)를 사용하여 최종적으로 제거한 후 4°C에서 냉장 보관하였다.

2.3. 정유의 항산화 활성 평가

2.3.1. DPPH 라디칼 소거활성 측정

1,1-diphenyl-2-picrylhydrazyl (DPPH) 라디칼 소거활성은 Blois (1958)와 Sharma (2009)의 방법을 변형하여 평가하였다. DPPH를 95% ethanol (Samchun, Korea)에 0.1 M로 용해시킨 후 정유와 1:1로 혼합하였다. 정유의 최종 농도가 3, 2, 1, 0.5, 0.25, 0.125 mg/mL가 되도록 맞추고 암실에서 30분간 반응시킨 후 UV-Vis spectrophotometer (Shimadzu uv-1601 pc spectrophotometer, kyoto, Japan)를 사용하여 517 nm의 흡광도를 측정하였다. DPPH 라디칼 소거율은 아래의 식 (1)을 통해 계산하였다. 또한 양성대조군인 trolox, ascorbic acid, tocopherol에 대하여 0-100 μM 범위의 검량곡선을 작성하였으며 이를 바탕으로 표준물질에 대한 식물정유의 당량값을 “μmol의 표준물질의 SC_{50}값/100 g의 식물정유의 SC_{50}값”의 단위로 산출하였다.

$$\text{DPPH radial scavenging ratio (\%)} = \{1-(As/Ac)\} \times 100 \quad (1)$$

As: Absorbance of the sample
Ac: Absorbance of the control

2.3.2. ABTS 라디칼 소거활성 측정

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) 라디칼 소거활성은 Re (1999)와 Konan (2016)의 방법을 변형하여 평가하였다. 7 mM의 ABTS 8 mL와 12.25 mM 과황산칼륨 수용액 (Samchun, Korea) 2 mL를 혼합 후 37°C 암실에서 16시간 동안 반응시켰다. 그 후 734 nm에서의 흡광도가 0.70 (±0.02)이 되도록 에탄올로 희석시켰다. 희석한 $ABTS^{\bullet+}$용액과 정유를 24:1로 정유의 최종 농도가 2, 1, 0.5, 0.25, 0.125 mg/mL이 되도록 맞추고 37°C 암실에서 5분간 반응시킨 후 UV-Vis spectrophotometer를 사용하여 734nm의 흡광도를 측정하였다. ABTS 라디칼 소거율은 아래의 식 (2)을 통해 계산하였다. 또한 양성대조군인 trolox, ascorbic acid, tocopherol에 대하여 0-100 μM 범위의 검량곡선을 작성하였으며 이를 바탕으로 표준물질에 대한 식물정유의 당량값을 "μmol의 표준물질의 SC_{50}값/100 g의 식물정유의 SC_{50}값"의 단위로 산출하였다.

$$\text{ABTS radical scavenging ratio (\%)} = \{1-(As/Ac)\} \times 100 \quad (2)$$

As: Absorbance of the sample
Ac: Absorbance of the control

2.3.3. TLC-DPPH(ABTS) 항산화 물질 분석

식물정유를 n-헥산:에틸아세테이트 (8:1)의 용매로 전개시킨 thin layer chromatography (TLC) 판 (TLC silica gel 60 F_{254}, merck, 20 × 20 cm, 0.2 mm thickness)에 DPPH 라디칼 용액 또는 ABTS 라디칼 용액을 분무하여 발색되는 활성물질군을 시각화하였다. 시각화된 TLC 판을 기준으로 전개 용매의 이동 거리에 대한 활성물질군의 이동 거리의 비(R_f값)를 계산하였다. 동일한 전개용매를 사용하여 prep. TLC (TLC silica gel 60 F_{254}, merck, 20 × 20 cm, 0.2 mm thickness)를 수행하고 R_f값을 기준으로 활성물질군이 흡착된 실리카겔에서 에틸아세테이트를 사용하여 해당 물질을 추출하였다. 추출물질의 정량, 정성분석을 위하여 GC/MS 성분분석을 실시하였다.

2.4. 선별 정유의 항미생물 효과

2.4.1. 병원성 미생물 생장억제능 활성 측정

식물정유의 항미생물 효과를 평가하기 위해 디스크 확산법을 Clinical and Laboratory Standards Institute (CLSI, USA)에서 제시한 규정에 따라 진행하였다. 1×10^6 CFU/mL (0.5 McFarland turbidity standard)의 농도로 만들기 위하여 UV-vis spectrometer (Shimadzu, Japan)를 통해 증류수로 *C. albicans*을 희석하여 530 nm에서 0.12~0.15 흡광도로 맞추었다. 희석된 *C. albicans* 접종물을 Mueller-Hinton agar supplemented with 2% glucose and methylene blue (MH-GMB, 0.5 μg/ml)배지에 접종한 뒤 100, 50 mg/mL 농도의 정유 20 μL를 각각 흡수시킨 직경 8 mm의 paper disc를 올려둔 후 26~28°C에서 4일간 배양하였다. 디스크 주변에 형성된 생장억제환의 지름을 측정하고 디스크 중앙부와 생장억제환 경계부분을 채취하여 Scanning electron microscope (SEM)을 이용하여 *C. ablicans*의 세포의 형태 변화를 관찰하였다.

2.4.2. MIC 측정

*C. albicans*의 Minimum Inhibitory Concentration (MIC)를 확인하기 위해 CLSI에서 제시한 규정에 따라 미량희석법을 진행하였다. 1×10^6 CFU/mL (0.5 McFarland turbidity standard)의 *C. albicans*을 1000배 희석하여 1×10^3 CFU/mL로 조정하였다. 96-well plate에 YB broth 배지를 100 μL씩 분주하고 정유를 농도별로 100 μL분주했다. 그 후 최종적으로 희석된 *C. albicans* 접종물을 100 μL씩 넣어준 뒤 26~28°C에서 4일간 배양하고 MIC를 측정하였다.

2.4.3. *C. albicans* 형태학적 분석

SEM은 Insuan & Chahomchuen (2020)에 제안된 방법을 변형하여 측정하였다. *C. albicans*를 SDA에 접종시킨 뒤 100 mg/mL 농도의 정유를 20 μL 흡수시킨 8 mm paper disc를 올려 26~28°C에서 4일간 배양하였다. 디스크 주변에 형성된 생장억제환 중앙부와 경계부위를 채취하여 carbon tape을 이용하여 aluminum stub에 부착시킨 후 2% osmium tetroxide vapor로 24시간 처리하여 *C. albicans*를 고정시켰다. Gold sputter을 이용하여 2분 동안 금으로 코팅시킨 후 SEM (Supra 55vp,

Carl Zeiss, Germany)을 이용하여 10,000~20,000배의 배율로 *C. albicans*의 표면적 변화를 관찰하였다.

2.4.4. TLC Bioassay 분석

본 연구에서 사용된 정유는 다양한 유기화학물질들의 혼합물이다. 이러한 성분들 중 항미생물 활성을 나타내는 주요성분들을 검출하기 위해 TLC bioassay 분석을 진행하였다. 식물정유를 n-헥산:에틸아세테이트(8:1)의 용매로 TLC 판에 전개시키고 UV 254 nm조건에서 관찰하여 활성 구역을 나누었다. 전개시킨 TLC 판을 전개 방향에 따라 절단하고 *C. albicans* 접종 후 5분이 경과된 고체 배지에 얹어주었다. 26~28℃에서 4일간 배양시킨 후 생장억제 구역을 확인하여 활성이 있는 부분과 동일한 R_f값을 가지는 분획 군을 prep. TLC 상에서 추출한 뒤 GC/MS 성분분석을 실시하였다.

2.5. GC/MS 분석

본 실험에서 시행된 GC/MS 성분분석은 tridecane을 내부표준물질로 하여, 모두 다음의 조건에 따라 성분분석을 진행되었다. GC (model-Agilent 7890, USA)분석을 위해 DB-5 column (25 m × 0.32 × 0.52 μm)를 사용하였다. Carrier gas는 헬륨을 사용하였고 온도 조건은 injector 260℃, detector 280℃, oven 온도는 초기농도 50℃에서 5분간 유지시킨 후 5℃/min씩 상승시켜 최종온도를 250℃까지 올린 후 10분간 유지시켜서 분석하였다. Mass spectrometry는 model-Agilent 5937을 사용하여 EI mode로 분석하였다. 얻어진 시료 피크의 mass data와 표준 library data (Willy 7th ed)와 비교하여 피크의 화합물 구조를 동정하였다.

3. 결과 및 고찰

3.1. 정유의 항산화 효과

3.1.1. DPPH 라디칼 소거 활성

육계나무 (*C. loureirii* Nees), 향나무 (*J. chinensis*), 산초나무 (*Z. schinifolium* S. et Z.), 초피나무 (*Z. piperitum*), 사철쑥 (*A. capillaris* Thunb.), 온주밀감 (*C. unshiu*), 사두감 (*C. pseudogulgul* hort.) 정유의 항산화 효과를 알아보기 위해 DPPH 라디칼 소거활성을 측정하였으며 그 결과는 Fig. 1과 같다. DPPH 라디칼 소거능은 flavonoids와 phenolic acids와 같은 페놀성 물질에 대한 항산화 지표로서 환원력이 클수록 라디칼 소거활성이 높은 것으로 알려져 있다(Kang *et al*., 1995). 대상 식물정유 7종 중 온주밀감과 육계나무 정유가 농도 3 mg/mL에서 DPPH 라디칼 소거능이 각각 95%, 94%로 사두감 정유 24%, 사철쑥 정유 91%, 초피나무 정유 23%, 향나무 정유 83%, 산초 정유 60%에 비해 높은 DPPH 라디칼 소거 활성을 나타냈다.

현재 항산화제로 사용되고 있는 trolox (수용성, 비타민 E 유사체), ascorbic acid (비타민 C), tocopherol (비타민 E)를 양성대조군으로 하여 DPPH 라디칼 소거 활성이 50%가 되었을 때의 농도 값을 의미하는 SC_{50}값과 표준물질 대비 당량값을 구하였으며, 항산화 양성대조군과 대상 식물정유의 SC_{50}값에 대한 결과는 Table. 2와 같다. 식물정유들의 SC_{50}값은 사철쑥 정유 0.82 mg/mL, 향나무 정유 1.25 mg/mL, 산초 정유 1.98 mg/mL, 사두감 정유 7.41 mg/mL, 초피나무 정유 8.42 mg/mL로 높은 값을 가지는 것에 비해 온주밀감과 육계나무 정유의 SC_{50}값은 0.10 mg/mL, 0.09 mg/mL로 다른 정유에 비해 낮은 값을 나타냈다. 또한 표준물질 대비 당량값을 비교해 봤을 때도 온주밀감 정유는 trolox 4.80, ascorbic acid 3.28, tocopherol 0.37의 당량값을 가지며, 육계나무 정유는 trolox 5.35, ascorbic acid 3.65, tocopherol 0.42의 당량값으로 다른 식물정유 5종보다 더 높은 값을 가진다. 이와 같은 결과들로 보았을 때 온주밀감과 육계나무 정유가 높은 항산화 활성을 가지는 것으로 사료된다. 이는 선행연구와 비교해 보았을 때도 온주밀감 정유는 DPPH 라디칼 소거활성이 최대 95%를 보였으며 육계나무 정유 또한 SC_{50}값이 0.073 mg/mL로 본 실험과 비슷한 양상을 나타냈다(Kim, 2013; Yang *et al*., 2012).

3.1.2. ABTS 라디칼 소거 활성

식물정유 7종에 대한 항산화 효과를 알아보기 위해 DPPH 라디칼 소거활성을 측정과 함께 ABTS 라디칼 소거 활성을 측정하였다. 그 결과는 Fig. 2과 같다. 대상 식물정유 7종 중 ABTS 라디칼 소거능이 농도 3 mg/mL에서 온주밀감 정유 95%, 육계나무 정유 96%, 향나무 정유 97%로 사두감 정유 80%, 사철쑥 정유 37%, 초피나무 정유 78%, 산초 정유 91%에 비해 높은 ABTS 라디칼 소거 활성을 나타냈다.

각 대상 식물정유에 대해 SC_{50}값과 항산화 표준물질인 trolox, ascorbic acid, tocopherol 대비 당량값을 구하였으며, 그 결과는 Table. 3와 같다. 식물정유들의 SC_{50}값은 향나무 정유 0.08 mg/mL, 산초 정유 0.25 mg/mL, 사두감 정유 0.10 mg/mL, 초피나무 정유 0.29 mg/mL, 온주밀감 정유 0.09 mg/mL, 육계나무 정유 0.06 mg/mL, 사철쑥 정유 4.05 mg/mL의 값으로 향나무와 온주밀감, 육계나무 정유에서 낮은 SC_{50}값을 나타냈다. 또한 표준물질 대비 당량값은 향나무 정유는 trolox 4.33, ascorbic

acid 2.26, tocopherol 2.97의 당량값을 가지며, 온주밀감 정유는 trolox 2.69, ascorbic acid 2.05, tocopherol 3.92의 당량값, 육계나무 정유는 trolox 3.94, ascorbic acid 3.01, tocopherol 5.75의 당량값으로 다른 식물정유 4종보다 더 높은 값을 가진다. 이와 같은 결과들로 보았을 때 향나무, 온주밀감, 육계나무 정유가 높은 항산화 활성을 가지는 것으로 사료된다.

3.1.3. GC/MS 성분 분석 및 TLC 분석

육계나무, 온주밀감 정유의 성분분석 결과는 Table 4와 같다. 육계나무 정유는 linalool (21.27%), α-citral (8.62%)이 주성분을 이루었으며, 온주밀감 정유는 limonene (69.87%), γ-terpinene (6.83%)이 주성분을 이루었다. 이는 선행연구에서도 육계나무 정유에서 linalool이 검출되었으며, 온주밀감 정유에서는 limonene, γ-terpinene의 성분들이 검출되었다(Xiao et al., 2013; Seshadri et al., 2020). 육계나무, 온주밀감 정유를 n-헥산:에틸아세테이트 (8:1)의 용매로 TLC 판에 전개시키고 Fig. 1과 같이 구역을 나눈 다음 R_f값을 지정했다.

3.1.4. 정유의 항산화 성분분석

DPPH 라디칼 소거능 평가와 ABTS 라디칼 소거능 평가 결과를 통해 SC_{50}값이 낮아 항산화 효과가 있다고 사료되는 온주밀감, 육계나무 정유를 선별하였으며 온주밀감 정유는 온주밀감B. 육계나무 정유는 육계나무C, D, E에서 항산화 활성을 나타냈다. 항산화 활성이 있는 부분을 GC/MS 성분분석 결과는 Table 5와 같다. 온주밀감B에서는 β-myrcene, linalool, elemol 등의 성분들이 검출되었다. 육계나무에서는 cinnamyl acetate, eucalyptol, linalool, citral이 주성분을 이루었으며 육계나무C에서는 eucalyptol, α-Citral, geranyl acetate, 육계나무D에서는 linalool, α-citral, 육계나무E에서는 linalool, β-citral, α-citral, cinnamyl acetate의 성분들이 검출되었다.

3.2. 정유의 항미생물 효과

3.2.1. 병원성 미생물 생장억제능 활성 측정

병원성미생물이 접종된 배지에 식물정유를 흡수시킨 8 mm paper disc의 주변의 생긴 생장억제환의 직경이 클수록 식물정유가 항미생물 활성이 있다고 알려져 있다. 식물정유 7종 중 뛰어난 라디칼 소거능을 보였던 온주밀감, 육계나무 정유의 *C. albicans*에 대해 디스크 확산법을 진행하였다. Fig. 4(a)와 같이 육계나무 정유 100 mg/mL 농도에 대하여 생장억제환이 형성되었으나 온주밀감 정유의 경우 100 mg/mL농도와 50 mg/mL농도에서 생장억제환이 관찰되지 않았다. 육계나무 정유의 생장억제환은 100 mg/mL에서는 44.9±8.9 mm, 50 mg/mL는 33.4±5.5 mm의 직경만큼 균주의 생장을 억제하였다. 이를 통해 온주밀감 정유는 *C. albicans*의 생장을 억제하지 못하나, 육계나무 정유의 경우 *C. albicans*에 대하여 항미생물 활성이 있음을 확인하였다.

3.2.2. MIC 측정

*C. albicans*를 식물정유에 노출시킨 뒤 26~28℃에서 4일간 배양시킨 뒤 육안으로 혼탁도를 비교하고 Minimum Bactericidal Concentration (MBC)와 MIC를 측정하였다. *C. albicans*에 대해서 육계나무 정유는 1.25 mg/mL로 낮은 MIC값을 나타냈지만 온주밀감 정유는 5 mg/mL로 높은 MIC값을 가진다. MBC 또한 육계나무 정유에서는 1.25 mg/mL값을 가졌으며 온주밀감 정유는 5 mg/mL 값을 가졌다. 육계나무 정유는 항미생물 활성이 우수하다고 알려진 편백나무와 삼나무, 소나무 정유와의 비교에도 높은 활성을 나타낸다. 편백나무, 삼나무 소나무 정유 모두 *C. alibicans*에서 2.18 mg/mL 이상의 MIC값을 가졌으며, *Candida krusei*, *Candida glabrata*, *Candida tropicalis*, *Candida pseudotropicalis*의 칸디다속 균주들에 대한 MIC값도 2.18 mg/mL 이상으로 육계나무 정유보다 더 낮은 항미생물 활성을 보였다(Lee *et al*., 2001; Lee *et al*., 2009). 이는 육계나무 정유는 *C. albicans*에 대해서 높은 항미생물 활성을 가지지만 온주밀감 정유는 *C. albicans*에 대한 항미생물 활성이 낮은 것으로 판단된다.

3.2.3. 균주의 형태학적 변화

식물정유들이 *C. albicans*의 생장을 저해하는 표면적인 현상을 시각화하기 위한 SEM 사진은 Fig. 5와 같다. 정유의 배지 내 확산 효과에 따른 생장정도의 차이를 비교하기 위하여 *C. albicans*의 대조군 사진 (Fig. 5(control))와 생장억제환의 경계부분과 생장억제환 중앙부로 나누어서 SEM 사진을 비교하였다 (Fig. 5(a,b,c,d)). 온주밀감 정유 처리 균주의 경우 생장억제환이 생성되지 않아 paper disc가 접촉된 부분의 균주를 채취하여 SEM 분석을 진행하였다. 생장억제환이 생성되지 않았던 온주밀감 정유 처리 시료의 경계부에서 채취한 균주의 경우 무처리 균주의 대조군 사진과 같이 표면이 매끈하고 생장이 억제되지 않음을

확인하였다 (Fig. 5(b)). 한편, paper disc를 떼어내고 채취한 부분의 경우 균이 거의 관찰되지 않았으며, 이는 disc의 접촉으로 인해 균이 자라지 않았기 때문으로 사료된다. 항미생물 활성을 보인 육계나무 정유에서 *C. albicans*의 세포벽이 파괴되고 수축되어 있는 모습을 관찰하였다. 이는 정유에 의해 세포벽이 파괴되고 균체 내부와 외부의 농도차이에 의해 내부 세포 구성기관들이 탈락하여 세포벽의 손상이 일어났기 때문으로 사료된다. 생장억제환 경계부위에는 손상되지 않은 *C. albicans*와 손상된 *C. albicans*가 함께 보이는 모습을 볼 수 있으며, 육계나무 정유를 처리한 생장억제환 내부의 SEM 사진인 Fig. 5(c)에서 *C. albicans* 세포 간 결합이 없이 서로 분리되고 수축되어 있는 모습이 관찰되었다.

3.2.4. TLC bioassay

TLC 판의 생장억제환을 확인한 결과 육계나무 정유의 육계나무 C, D, E 부분에서 생장억제환이 나타났다. 해당 부분을 GC/MS로 분석결과는 Table 6와 같다. 육계나무 정유의 원시료에서는 cinnamyl acetate, eucalyptol, linalool, citral이 주성분을 이루었으며 육계나무C에서는 eucalyptol, α-citral, geranyl acetate, 육계나무D에서는 linalool, α-citral, 육계나무E에서는 linalool, β-citral α-citral, cinnamyl acetate의 성분들이 검출되었다. 육계나무 정유의 eucalyptol는 *S. aureus*에 항미생물 활성을 보인다는 선행연구 결과가 있으며, 육계나무C, D, E에서 모두 검출된 citral 또한 항미생물 활성이 있다는 선행연구가 있다 (Karlović *et al*., 2000; Saddiq *et al*., 2010). Citral은 *C. albicans*에 대한 생장억제능 활성 측정을 통한 생장억제환이 40 mm 이상의 직경을 가졌으며, 이는 본 실험에서 진행한 육계나무 정유에 대한 *C. albicans*의 생장억제환 직경과 유사한 수치를 나타내며 육계나무 정유가 높은 항미생물 활성을 나타냄을 의미한다. 뿐만 아니라 다른 칸디다속 균주에도 우수한 항미생물 활성을 보였는데 *Candida parapsilosis* 27.5 mm, *C. krusei* 19.7 mm, *C. tropicalis* 32.6 mm의 생장억제환 직경을 가졌다(Silva *et al*., 2008). Cinnamyl acetate가 주성분을 이루는 실론계피나무 정유 또한 *Candida albicans*에 0.12 mg/mL의 MIC값을 가져 항미생물 활성을 보였다(Unlu *et al*., 2010; de Lima Carvalho *et al*., 2018). Geranyl acetate는 칸디다속 균주에 항미생물 활성을 나타낸다는 선행연구가 있으며, *C. krusei*에는 20 μl/mL의 MIC값, *C. parapsilosis*는 5 μl/mL의 MIC값, *C. guillermondii*는 1.25 μl/mL의 MIC값을 가졌다. 뿐만 아니라 *T. rubrum*과 *T. mentagrophytes*는 0.32 μl/mL의 MIC값을 가지며, *A. fumigatus*는 20 μl/mL의 MIC값을 가지는 등 다른 여러 균주들에도 뛰어난 항미생물 활성을 보인다(Goncalves *et al*., 2012). Eucalyptol, citral, cinnamyl acetate, geranyl acetate 성분들이 항미생물 활성을 나타내는 것으로 사료된다. 육계나무 정유의 항산화와 항미생물 활성부위에 검출된 linalool은 항미생물 활성이 나타나지 않았던 온주밀감에도 검출되었다. 선행연구에서 진행된 *C. albicans*에 대한 linalool의 디스크 화산법과 MIC값은 12 mm의 생장저해환이 나타났고, 1.23 mg/mL의 MIC값으로 높은 항미생물 활성을 나타냈다(Herman *et al*., 2016). *C. albicans* 뿐만 아니라 다른 균주에서도 좋은 항미생물 활성을 보였는데 *Aggregatibacter actinomycetemcomitans*는 0.1 mg/mL의 MIC값, *Porphylomonas gingivalis*는 0.8 mg/mL의 MIC값, *Prevotella intermedia*은 0.2 mg/mL의 MIC값을 가지는 등 여러 균주들에서도 좋은 항미생물 활성을 나타냈다(Hsu *et al*., 2013). 온주밀감에도 항미생물 활성이 있는 linalool이 검출되었지만 온주밀감 정유의 구성성분 중 0.72% 미량 존재하여 항미생물 활성을 보이지 않는 것으로 사료된다.

4. 결 론

본 연구에서는 식물정유 7종의 항산화 평가를 통해 우수한 항산화 활성을 지닌 육계나무, 온주밀감 정유를 선정하였고, 이들 정유의 *C. albicans*에 대한 항미생물활성을 구명하였다. 식물정유 7종 중 우수한 항산화 활성을 나타낸 온주밀감과 육계나무 정유의 TLC-DPPH (ABTS)를 진행하였으며 GC/MS 분석을 통해 유효성분을 구명하였다. 온주밀감 정유는 linalool, 육계나무 정유는 eucalyptol, α-citral, β-citral, linalool이 주성분을 이루었다. 우수한 항산화 활성을 보인 온주밀감과 육계나무 정유의 *C. albicans*에 대한 항미생물 평가 결과 온주밀감 정유는 항미생물 활성을 보이지 않았지만 육계나무 정유에서는 MIC값이 1.25 mg/mL로 높은 항미생물 활성을 나타냈다. 이는 라벤더 (*Lavandula officinalis*), 로즈마리 (*Rosmarinus officinalis*), 유칼립투스 (*Eucalyptus globulus*) 정유의 *C. albicans*에 대한 MIC 값이 5 mg/mL를 나타낸 선행연구와 비교했을 때도 높은 값을 의미한다(Tampieri et al., 2005). TLC-bioassay를 통해 육계나무C, D, E 부분에서 항미생물 활성을 나타냄을 확인했으며, 활성 분획에서 eucalyptol, citral, geranyl acetate, cinnamyl acetate이 검출되었다. 결론적으로 항산화 활성과 *C. albicans*에 대한 항미생물 활성이 좋은 육계나무 정유는 차후 피부 문제를 해결할 수 있다고 평가되며 특히 육계나무 C, D, E의 성분들은 항산화와 항미생물 두 가지 효과가 있는 제품으로서의 개발 가능성이 충분하다고 사료된다.