JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2009. Vol. **3**(2), p. 429-434

Antimicrobial Effect of *Lindera erythrocarpa* Essential Oil Against Antibiotic-Resistant Skin Pathogens

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(Received: 17 May 2009; accepted: 20 June 2009)

Due to increases in antibiotic resistance, there is mounting interest in essential oils as alternatives to synthetic drugs, particularly against microbial agents. In the present study, we evaluated the chemical composition and the antibacterial activity of the *Lindera erythrocarpa* oil (LEE). *L. erythrocarpa* was collected from Jeju Island, south of Korea, and the essential oil was examined by GC/MS and bacteriological tests. Thirty-one compounds representing 91.85% of LEE was identified. The major constituents of LEE were nerolidol (26.93%) and beta-caryophyllene (13.24%). Other chemical components included methyl cinnamate (8.54%), alpha-humulene (8.48%), geranyl acetate (7.82%), alpha-farnesene (6.20%) and alpha-pinene (3.48%). The antibacterial activities of LEE against skin pathogens have not previously been reported. Thus, we determined the anti-bacterial activities of LEE using the disk diffusion method and the minimum inhibitory concentration (MIC) values. In this study, LEE showed excellent antibacterial activities against drug-susceptible and -resistant skin pathogens such as *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Malassezia furfur*, which are acne-causing bacteria. The MIC of LEE against drug-susceptible and -resistant skin pathogens ranged from 0.15 to 20.0 μL/mL.

Key word: Chemical composition, Essential oil, *Lindera erythrocarpa*, Skin pathogen.

Acne vulgaris, a skin disease, is a chronic inflammatory disorder of pilosebaceous follicles that affects more than 85 percent of adolescents and young adults[1]. Its pathogenesis is related to sebum production by the sebaceous glands, hyperkeratinization of the follicle, bacterial colonization of the sebaceous follicle, and the release of inflammatory mediators[2,3]. In bacterial colonization of the follicle, the three main microorganisms isolated from the skin surface and ducts of the sebaceous glands are *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Malassezia furfur*. *P. acnes* is the most important of these microorganisms, which are present as part of the resident commensal flora. On normal undamaged human skin, these microorganisms are considered non-pathogenic. However, under certain conditions, they can cause opportunistic infections that may

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present as serious skin problems[4]. As therapeutic agents for acne vulgaris, antibiotics such as tetracycline, erythromycin, and clindamycin are usually employed to inhibit the growth of skin pathogens. However, the widespread use of antibiotics in acne vulgaris has resulted in the emergence of antibiotic-resistant pathogens that can negatively impact public health through an increased incidence of treatment failure and more severe disease[5]. Therefore, many researchers are increasingly turning their attention to alternative medicines to develop better drugs against drug-resistant pathogens.

Lindera erythrocarpa Makino is one of the Lauraceae woody plants, which are widely distributed in Asia, including Korea, Taiwan, Japan, and China; the fruits of these plants have been used as ingredients in folk analgesic, digestive, diuretic, antidote and antibacterial remedies[6-8]. Recent reports have demonstrated that *L. erythrocarpa* has several medicinal functions, including anti-inflammatory and anti-tumor effects[8,9]. In addition, it exhibits chitin synthase 2 inhibitory activity and antimicrobial activity against several fungi[10]. However, the chemical composition and anti-bacterial activities of LEE against drug-resistant skin pathogens have not been described. In this paper, we demonstrate that LEE has antibacterial effects against skin pathogens. To the best of our knowledge, this is the first report demonstrating the *in vitro* anti-bacterial activities of LEE against drug-susceptible and -resistant skin pathogens and providing a scientific basis for their use in human skin health.

MATERIAL AND METHODS

Plant material and extraction

The plants were collected from Jeju Island, South Korea, in August 2008. Voucher specimens were identified by Dr. G. Song and deposited at the JeJu Biodiversity Research Institute (JBRI) (Jeju, South Korea). The essential oil of *L. erythrocarpa* was extracted by hydrodistillation. Briefly, approximately 1 kg of fresh *L. erythrocarpa* leaves was immersed in 4 L of distilled water in a 5-L three-neck flask for 6 h at atmospheric pressure. The essential oil was analyzed by GC/MS on a Hewlett-Packard mass spectrometer 5975 at 70 eV coupled to an HP6890N GC equipped with a DB1-HT column (30 m × 0.32 mm × 0.1 ìm). The oven temperature was programmed from 40 to 100 °C at a rate of 2 °C /min, 100 to 230 °C at a rate of 5 °C /min, and held at 230 °C for 5 min (71 min analysis time). The injector and detector temperatures were 240 and 280 °C, respectively. The flow rate of the carrier gas (He) was 1.5 ml/min and the split ratio was 1:10. For the injection (splitless), 10 µl of essential oil was diluted in 500 µl of CH_2Cl_2, and 1 µl of this diluted sample was injected. The volatile constituents were identified on the basis of their mass spectra, which were compared to those in the literature. The retention indices were calculated using a homologous series of *n*-alkanes C_6-C_{25}. The peak areas of individual compounds were related to total peak areas of compounds detected by GC.

Microorganisms

Three Gram-positive bacterial species that are each involved in acne, *S. epidermidis* CCARM 3709, *S. epidermidis* CCARM 3710, *S. epidermidis* CCARM 3711, *P. acnes* CCARM 0081, *P. acnes* CCARM 9009, *P. acnes* CCARM 9010 and *Malassezia furfur* KCCM 12679, were selected as test microorganisms according to their pathological capacity. They were obtained from the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Seoul, Korea) and Korea Collection Center of Microorganism (KCCM, Seoul, Korea). We also used *Candida albicans* KCCM 11282 as a test pathogen. *Propionibacterium* strains were cultured at 37 °C for 48 h in GAM broth (Nissui Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions before the assay. *S. epidermidis* were cultured at 37 °C for 24 h with *Corynebacterium* media (10.0 g casein peptone, 5.0 g yeast extract, 5.0 g glucose, and 5.0 g NaCl per liter). *M. furfur* was grown at 37 °C for 24 h on YM agar containing 1% olive oil. *C. albicans* was also cultured at 37 °C for 24 h in YM broth.

Disk diffusion assay

The inhibitory effects on test bacteria were determined by the disk diffusion method. The cell suspension was adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland n. 0.5 standard (1.5×10^8 cells/mL). The disks (Φ 8.0 mm) were prepared and

the essential oils, diluted in ethanol to the test concentrations, were added to the disks (20 µL); an equal volume (20 µL) of ethanol was used as a control. The inoculated plates were incubated at 37 °C for 48 h under anaerobic conditions for *Propionibacterium* sp. (BBL GasPak System). Other pathogens were incubated at 37 °C for 24 h under aerobic conditions. After incubation, the diameter of the inhibition zone was measured with calipers.

Minimum inhibitory concentration (MIC) determination

The micro-dilution susceptibility assay was performed using the NCCLS (National Committee for Clinical Laboratory Standards) method for determination of the minimum inhibitory concentration. A stock solution of essential oil was prepared in 10% dimethylsulfoxide (DMSO) and serially diluted to 0.1 to 40 µL/mL. The 96-well plates were prepared by dispensing 95 µL of culture broth, 100 µL of essential oil and 5 µL of the inoculants into each well. A positive control (containing inoculum but no essential oil) and a negative control (containing essential oil but no inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at proper temperature and incubation times. The MIC was defined as the lowest concentration of the compound that inhibited microorganism growth.

RESULTS AND DISCUSSION

Many skin diseases have been treated with herbal remedies throughout human history. Indeed, herbs continue to play a major role in primary health care as therapeutic remedies in many developing countries[11]. There is a continuous and pressing need to discover novel antibacterial candidates with diverse chemical structures and novel mechanisms of action against new and re-emerging skin pathogens [12]. As part of our ongoing alternative medicine program, we directed our attention toward the identification of essential oils that combine a relatively narrow spectrum of activity against skin pathogens, including tetracycline-, erythromycin- and clindamycin-resistant strains, for potential topical applications in patients with mild to moderate inflammatory skin disease such as acne vulgaris. Here we report the chemical composition and antibacterial activities of LEE against drug-resistant skin pathogens. The essential oils were obtained by hydrodistillation in a Clevenger-type apparatus from *L. erythrocarpa* leaves. The identified compounds and qualitative and quantitative analytical results by GC and GC/MS are shown in Table 1, according to their elution order on a DB1-HT column. The GC-MS analysis of LEE led to identification of 31 components, representing 91.85% of the total oil constituents. The major constituents of LEE were nerolidol (26.93%) and beta-caryophyllene (13.24%). Other chemical components included methyl cinnamate (8.54%), alpha-humulene (8.48%), geranyl acetate (7.82%), alpha-farnesene (6.20%) and alpha-pinene (3.48%). A portion (8.15%) of the total composition was not identified. The antimicrobial activities of essential oils from many plants have been recognized; only recently have their effects on skin health been confirmed. The plant source, harvest season, stage of development, extraction technique, drying conditions of plant material, tested bacteria and antimicrobial methodology are all factors that influence the antimicrobial activity and must therefore be taken into account whenever antimicrobial assays are performed with these oils. The *in vitro* antimicrobial potential of LEE against the tested skin pathogens was quantitatively assessed by the presence or absence of inhibition zones and MIC values. The disk diffusion method was employed as a susceptibility screening test to evaluate the activity of LEE against eight skin pathogens. The results are presented in Table 2. The disk diffusion assay is generally used for preliminary screening of antibacterial activities prior to more detailed evaluation. Furthermore, this assay is limited to the generation of preliminary quantitative data only, since the hydrophobic nature of most essential oils prevents their uniform diffusion. Therefore, an emulsifying agent such as DMSO should be used to assure contact between the tested bacteria and the possible antibacterial candidate[13-15]. As seen in Table 2, LEE exhibited varying degrees of bacterial growth inhibition against the skin pathogens tested. They showed excellent *in vitro* antimicrobial activity against the all the tested

skin pathogens, including eight Gram-positive bacteria and two yeast strains, with zones of inhibition of 11-20 mm in diameter. The antibacterial activities of LEE was further evaluated by determining the MIC, which is the lowest concentration yielding no growth. These two oils were most active against antibiotic-susceptible *S. epidermidis* CCARM 2709 (MIC value of 0.15 µL/mL); however, tetracycline-resistant *S. epidermidis* CCARM 3711 showed less susceptibility to the essential oils (MIC values of 20 µL /mL).

Beta-caryophyllene, alpha-humulene, geranyl acetate, alpha-pinene, and beta-pinene are representative components in some oils that exhibit antimicrobial activity[16-18]. Nerolidol and alpha-pinene also exhibit antifungal activity[19,20]. However, it is difficult to attribute the activity of a complex mixture to a single constituent. Major or trace compounds might give rise to the antibacterial activity exhibited. Possible synergistic and antagonistic effects of compounds in the LEE should also be considered. To our knowledge, this is the first study to provide data that LEE possess antibacterial activities. Also, the chemical composition of these essential oils is

Table 1. Chemical composition (%) of *L. erythrocarpa* essential oil

RT (min)	RI	Constituents	Peak area (%)
5.103	917.6	α-pinene	3.48
5.472	926.7	camphene	1.99
6.497	952.0	β-pinene	1.01
7.543	977.7	camphene	0.19
7.824	984.7	α-phellandrene	0.13
8.601	1002.7	cymene	0.28
8.854	1007.2	β-phellandrene	0.42
9.042	1010.5	limonene	0.34
9.846	1024.7	cis-β-ocimene	0.42
10.375	1034.0	trans-β-ocimene	0.45
13.063	1081.5	β-linalool	0.72
23.865	1254.3	bornyl acetate	2.53
27.999	1320.2	α-terpinene	0.22
28.878	1334.6	methyl cinnamate	8.54
29.781	1349.3	α-copaene	0.68
30.625	1363.1	geranyl acetate	7.82
30.801	1365.9	β-elemene	0.34
31.941	1384.5	β-Caryophyllene	13.24
32.591	1395.1	Germacrene D	0.18
33.533	1409.8	α-guaiene	0.17
33.803	1413.9	α-humulene	8.48
34.161	1419.4	Alloaromadendrene	0.20
35.384	1438.1	Germacrene D	1.43
36.321	1452.4	α-selinene	0.61
37.775	1474.5	α-farnesene	6.20
38.656	1488.0	δ-cadinene	1.18
41.416	1534.8	caryophyllene oxide	1.53
42.275	1550.0	nerolidol	26.93
44.870	1595.7	δ-cadinene	0.90
45.013	1598.3	aromandendrene	0.54
45.300	1605.6	α-cadinol	0.70

The GC/MS retention indices were calculated using a homologous series of *n*-alkanes C_6-C_{31}. Components were characterized based on library and literature searches and only those components showing matches exceeding 80% were selected. RI, retention index; RT, retention time.

described in detail. The oils obtained from *L. erythrocarpa* is quite interesting from a pharmaceutical standpoint because of their antibacterial properties against skin pathogens. For instance, *S. epidermidis, P. acnes* and *M. furfur* are known to worsen skin acne in humans and thus LEE may be good candidates for medicated acne care formulations. Further investigations are in progress to compare the activities of LEE and some of their constituents in order to identify plant substances for future antibacterial formulations.

There is increasing interest in medicinal plants as alternatives to synthetic drugs, particularly against microbial agents because of increasing antibiotic resistance. Essential oils of herbs and their components have many applications in alternative medicines, food, beverages, and cosmetics, as well as in the fragrance and pharmaceutical industries. However, the increased usage of essential oils worldwide has raised a number of concerns related to adverse health effects that need to be addressed. The current results, which demonstrate the potent antibacterial activity of LEE against drug-susceptible- and resistant skin pathogens, are noteworthy and justify the traditional use of these plants, but further investigations are necessary to evaluate the practical applicability of these remarkable antibacterial properties. Therefore, we suggest that LEE be further explored as potential therapeutic agents to promote skin health..

Table 2. Antimicrobial activity of the essential oil from *L. erythrocarpa* (LEE)

Strains	Drug-resistant patterns of skin pathogens (MIC; μg/mL)	Inhibition zones (mm)	MIC values (μL/mL)
S. epidermidis CCARM 3709	Susceptible	1.5	0.15
S. epidermidis CCARM 3710	Erythromycin (>32), Clindamycin (>16), Chloramphenicol (64)	1.4	2.5
S. epidermidis CCARM 3711	Tetracycline (>32)	1.2	20
P. acnes CCARM 0081	Susceptible	1.6	0.15
P. acnes CCARM 9009	Clindamycin (64)	1.6	0.15
P. acnes CCARM 9010	Clindamycin (64)	1.5	0.3
M. furfur KCCM 12679	-	1.1	5.0
C. albicans KCCM 11282	-	2.0	5.0

The inhibitory effect of LEE on test bacteria was determined by the disk diffusion method. Skin pathogens were grown at 37 °C for 24 h or 48 h in each media. Culture suspensions were adjusted by comparing against 4 McFarland. The disks (Φ 8.0 mm) were prepared and the essential oil, diluted in ethanol to the test concentration, was added to the wells (20 μL). The same volume of ethanol (20 μL) was used as a control. For determination of MIC, the microdilution broth susceptibility assay was used.

ACKNOWLEDGMENTS

The authors are grateful to Mr. W. H. Yang for technical assistance. This research was supported by the Regional Technology Innovation Program (RTI04-02-07), which is managed by the Ministry of Knowledge and Economy, Korea.

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