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Extracts and Essential Oil of *Ledum palustre* L. Leaves and Their Antioxidant and Antimicrobial Activities

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Abstract

The *in vitro* antimicrobial and antioxidant activities of the essential oil and methanol extracts of *Ledum palustre* L. were investigated. Using GC-MS analysis, we identified 37 compounds in the essential oil, constituting 87.58% of the total oil. There are several monoterpenes, of which sabinene is the major compound (16~17%). There are several oxygenated monoterpenes of which terpinen-4-ol (7.6%) and myrtenal (3.5%) are the main constituents. β-Selinene, a-selinene, y-elemene, a-caryophyllene are the main sesquiterpenes (2~6% range). The oil strongly reduced the diphenylpicrylhydrazyl radical (IC_{50}=1.56 μg/mL) formation and exhibited a hydroxyl radical scavenging effect in the Fe^{3+}-EDTA-H_2O_2 deoxyribose system (IC_{50}=2.7 μg/mL), and also inhibited the nonenzymatic lipid peroxidation of rat liver homogenate (IC_{50}=13.5 μg/mL). The polar phase of the extract showed antioxidant activity. The oil showed antimicrobial activity against *Streptococcus pneumoniae*, *Clostridium perfringens*, *Candida albicans*, *Mycobacterium smegmatis*, *Acinetobacter lwoffii* and *Candida krusei* while the water-insoluble parts of the methanolic extracts exhibited slight or no activity. This study confirms that the essential oil of *Ledum palustre* L. possesses antioxidant and low antimicrobial properties *in vitro*.

Key words: *Ledum palustre* L., antioxidant activity, antimicrobial activity, methanol extracts, essential oil, GC-MS

INTRODUCTION

It has long been recognized that naturally occurring substances in higher plants have antioxidant activity. Recently, there has been a growing interest in oxygen-containing free radicals in biological systems and their implied roles as causative agents in the etiology of a variety of chronic disorders. Accordingly, attention is focused on the protective biochemical functions of naturally occurring antioxidants in the cells of the organisms containing them. Antioxidants in oils are important for stabilizing free fatty acids (1-3). The antioxidant activity of phenols and other compounds present in oils has been well and widely studied by many researchers (4-6).

Ledum palustre L. is a shrub growing in north and central Europe, north Asia and America (7,8). It is reported to be used in allopathy and in homeopathy for the treatment of rheumatism, arthrosis, insect bites (9-11) and in folk medicine as a lactagog and abortifacient (12).

Previous studies have reported the presence of essential oils containing the sesquiterpene alcohols palustrol and ledol (13), sterols (14), flavonoids (15) and coumarins (5). Several trienes have been identified as compounds responsible for the odor (16). However, it has not been reported yet whether *Ledum palustre* L. exhibits antioxidant properties. The aim of this study was to evaluate the *in vitro* antioxidant and antimicrobial properties of the essential oil and the methanol extracts of *Ledum palustre* L.

MATERIALS AND METHODS

Collection of plant material and extraction of the essential oil

The herbal parts of *Ledum palustre* L. were collected in Beckdoo Mountain, North Korea, when July 2005. Specimens have been deposited at the Bio-MAX Institute of Seoul National University in South Korea. The dried and powdered aerial parts (300 g) were subjected to 3 hrs to water-distillation using a Clevenger-type apparatus to produce oil with a 0.6% (v/w) yield. Oil was dried over anhydrous sodium sulfate and, after filtration, stored at 4°C until tested and analyzed.

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GC-MS analysis

The analysis of the essential oil was performed using a Hewlett Packard 5890 II GC, equipped with a HP-5 MS capillary column (30 m×0.25 mm i.d., 0.25 μm) and a HP 5972 mass selective detector. For GC-MS detection, an electron ionization system was used with ionization energy of 70 eV. Helium was the carrier gas, at a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 220 and 290°C, respectively. Column temperature was initially at 50°C, then gradually increased to 150°C at a rate of 3°C/min, held for 10 min and finally increased to 250°C at 10°C/min. Diluted samples (1.0 μL, 1/100 in acetone) were injected manually and split less. The components were identified based on the comparison of their relative retention time and mass spectra with those in the NBS75K library data of the GC-MS system, literature data (4) and standards of the main components. The results were also confirmed by the comparison of the compounds elution order with their relative retention indices on non-polar phases reported in the literature (4).

Antioxidant activity

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and the extract or crude oil for hydroxyl radicals, which attack deoxyribose leading to the formation of thiobarbituric acid reactive substances (TBARS), generated from the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (17). The formed TBARS were measured by using the method described elsewhere (18). Experiments were carried out in triplicate. All reagents were prepared freshly. Percent inhibition (L) of deoxyribose degradation was calculated in following way:

$$L=(A_0-A_1/A_0)\times 100$$

where A_0 is the absorbance of the control reaction (containing all reagents except the test compound), and A_1 is the absorbance of the test compound. The IC_{50} value represented the concentration of the compounds, which caused 50% inhibition.

Inhibition of superoxide radicals: Superoxide radical generated by the xanthine-xanthine oxidase system was determined spectrophotometrically by monitoring its ability to reduce nitroblue tetrazolium (NBT) (15). Percent scavenging of superoxide was calculated from the optical density of the treated and control samples.

DPPH assay: Radical scavenging activity was determined by a spectrophotometric method based on the reduction of a methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described elsewhere (19). Tests were carried out in triplicate.

Inhibition of lipid peroxide formation: The reaction mixture contained 0.1 mL of 25% (w/v) rat liver homogenate in 40 mM, pH; 7.0 Tris-HCl buffer, 30 mM KCl, 0.16 mM ferrous iron, various concentrations of the extract, and positive controls; BHT, curcumin, 0.06 mM ascorbic acid in a final volume of 0.5 mL. As positive controls, BHT and curcumin had their own control reactions containing all related reagents except the test compounds. The mixture was then incubated at 37°C for 1 hr (20). The lipid peroxide formation was measured by using a method described elsewhere (18). The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compounds with those of controls not treated with the extracts. Calculations were done as mentioned in the hydroxyl radical scavenging method.

Antimicrobial activity

Microbial strains: The methanolic extracts (both water-soluble and water-insoluble parts) and the essential oil of *Ledum palustre* L. were individually tested for minimum inhibitory concentration (MIC test) against a panel of microorganisms including: *Staphylococcus aureus* ATCC #25923 and ATCC #29213, *Streptococcus pneumoniae* ATCC #49619, *Moraxella catarrhalis* ATCC #49143, *Bacillus cereus* ATCC #11778, *Acinetobacter lwoffii* ATCC #19002, *Enterobacter aerogenes* ATCC #13043, *Escherichia coli* ATCC #25922, *Klebsiella pneumoniae* ATCC #13883, *Proteus mirabilis* ATCC #7002, *Pseudomonas aeruginosa* ATCC #27853, *Clostridium perfringens* KUKENS-Turkey, *Mycobacterium smegmatis* CMM 2067, *Candida albicans* ATCC #10239 and *Candida krusei* ATCC #6258. Bacterial strains were cultured overnight at 37°C in Mueller Hinton agar (MHA), with the exception of *Streptococcus pneumoniae* (MHA containing 50 mL citrate blood/L) and *Clostridium perfringens* (in anaerobic conditions). Yeasts were cultured overnight at 30°C in Sabouraud dextrose agar.

Antimicrobial screening: Two different methods were employed for the determination of antimicrobial activities; agar well-diffusion method for the methanol extracts (water-soluble and water-insoluble parts) and agar disc diffusion method for the essential oil. MICs of the essential oil against the test organisms were determined by the broth microdilution method. All the tests were performed in duplicate and repeated twice. Modal values were selected.

Agar well-diffusion method: The water-soluble extracts were weighed and dissolved in phosphate buffer saline (PBS; pH 7.0~7.2), 10 mg/mL, water-insoluble parts were dissolved in dimethylsulphoxide (DMSO), 10 mg/mL.

Both extracts were filter-sterilized using a 0.45 μm membrane filter. Each microorganism was suspended in sterile saline and diluted at ca. 10^6 colony forming unit (cfu)/mL. They were "flood-inoculated" onto the surface of MHA. The wells (8 mm in diameter) were cut from the agar and 0.06 mL of extract solution was delivered into them. After incubation for 24 hr at 37°C, all plates were examined for any zones of growth inhibition, and the diameter of these zones were measured in millimeters.

Disc diffusion method : The disc diffusion method was employed for the determination of antimicrobial activities of the essential oil. Briefly, a suspension of the tested microorganism (0.1 mL of 10^8 cells per mL) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 15 μL of the oil or the fraction and placed on the inoculated plates. These plates, after staying at 4°C for 2 hr, were incubated at 37°C for 24 hr for bacteria and at 30°C for 48 hr for the yeasts. The diameters of the inhibition zones were measured in millimeters. Amikacin, clindamycin and ciprofloxacin were individually used as positive controls for bacteria.

Determination of minimum inhibitory concentration (MIC) : A broth microdilution broth susceptibility assay was used, as recommended by NCCLS, for the determination of MIC. All tests were performed in Mueller Hinton broth (MHB; BBL) supplemented with Tween 80 detergent (final concentration of 0.5% (v/v), with the exception of the yeasts (Sabouraud dextrose broth-SDB + Tween 80). Bacterial strains were cultured overnight at 37°C in MHA and the yeasts were cultured overnight at 30°C in SDB. Test strains were suspended in MHB to give a final density of 5×10^5 cfu/mL and these were confirmed by viable counts. Geometric dilutions ranging from 0.036 mg/mL to 72.00 mg/mL of the essential oil were prepared in a 96-well microtiter plate, including one growth control (MHB + Tween 80) and one sterility control (MHB + Tween 80 + test oil). Plates were incubated under normal atmospheric conditions at 37°C for 24 hr for bacteria and at 30°C for 48 hr for the yeasts. The MIC of amikacin, clindamycine and ciprofloxacine was individually determined in parallel experiments in order to control the sensitivity of the test organisms. The bacterial growth was indicated by the presence of a white "pellet" on the well bottom.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

Thirty-seven compounds were identified and constituted 87.57% of the total oil. The essential oil of *Ledum palustre* L. was characterized by a high number of monoterpenes, of which sabinene was the major compound (16~17%). There were also several oxygenated monoterpenes with terpinen-4-ol (7.6%) and myrtenal (3.5%) being the main constituents. β-Selinene, α-selinene, δ-elemene, α-caryophyllene were the main sesqui-terpenes (2~6% range).

The occurrence of a sesquiterpene with a Kovats' index higher than that of β-elemene (115 *Rt* units higher) and a mass spectrum identical to that of β-elemene was observed. Although it is named 'isomer of β-elemene' (stereoisomer) in Table 1, we can only identify a sesquiterpene. Unidentified oxygenated sesquiterpenes were

Table 1. Chemical composition of the essential oil from *Ledum palustre* L.

No.	Compound[1)]	*Rt*[2)]	Composition (%)
1	(*E*)-2-Hexenal	1225	0.09
2	Tricyclene	1011	0.22
3	α-Thujenets	1024	0.41
4	α-Pinene	1017	2.18
5	Camphene	1063	1.70
6	Sabinene	1121	17.84
7	β-Pinene	1105	2.9
8	Myrcene	1166	0.31
9	α-Phellandrene + Menthatriene	1163	0.18
10	α-Terpinene	1175	1.65
11	p-Cymene	1273	1.20
12	Limonene	1192	0.69
13	β-Phellandrene	1199	0.54
14	(*Z*)-β-Ocimene	1238	0.26
15	(*E*)-β-Ocimene	1257	0.09
16	γ-Terpinene	1245	3.26
17	Terpinolene	1285	0.61
18	*cis*-Pinene hydrate	1888	1.42
19	*trans*-Pinocarve	1652	2.65
20	Pinocarvone	1563	2.35
21	Terpinen-ol	1597	7.61
22	Myrtenal	1621	7.44
23	Myrtenol	1626	3.53
24	*trans*-Carveol	1755	0.50
25	*cis*-Carveol	1791	1.44
26	Cumin aldehyde	1779	1.25
27	Bornyl acetate	1576	3.37
28	β-Caryophyllene	1586	0.59
29	Humulene	1663	2.46
30	Germacrene-D	1705	0.47
31	β-Selinene	1714	6.44
32	α-Selinene	1720	1.98
33	iso-Furanogermacrene *Mr* 216 (*m/z*: 108)	1867	0.35
34	γ-Elemene	1816	3.35
35	Germacrone	2218	0.44
36	*Mr* 220 (*m/z*: 55,67,107,81,109)	2284	0.90
37	*Mr* 218 (*m/z*: 95,69,67,41,176)	2493	4.91

[1)]Compounds listed in order of elution from a HP-5 MS column.

[2)]Retention time (as minutes).

Table 2. Effects of methanolic extracts (water-soluble part) and essential oil from *Ledum palustre* L. on free radical (DPPH, superoxide and hydroxyl) formation and lipid peroxidation

Sample	IC50 (μg/mL)			
	DPPH	Superoxide	Hydroxyl	Lipid peroxidation
Extract	45.60±1.30	304.00±5.10	407.30±4.05	892.67±13.0
Essential oil	1.56±0.03	not tested	2.70±0.03	13.50±0.07
Curcumin	7.92±0.30	11.04±0.17	14.28±0.08	40.83±0.15
Ascorbic acid	3.90±0.15	1390.00±2.90	not tested	not tested
BHT	19.30±0.05	not tested	32.00±1.20	17.80±0.04

also present. Linalool (0.11%), δ-elemene (0.05%), α-copaene (0.05%), β-bourbonene (0.15%), β-elemene (0.21%), α-farnesene (0.18%), δ-cadinene (0.21%), guaiol (0.04%) and β-elemenone (0.47%) were also identified in a sample containing 10% germacrone.

Antioxidant activity

The antioxidant activity of *Ledum palustre* L. extract (water-soluble part) was examined by comparing it to the activity of three well characterized antioxidants (curcumin, ascorbic acid and BHT) by the following four in vitro assays; inhibition of DPPH radical and the oxygen radicals such as lipid peroxides, superoxides, and hydroxyl radicals. Since the water-insoluble part of the extract is partly soluble in aqueous test media and its color interfered the spectroscopic measurements, only the water-soluble portion and the essential oil could be tested for their antioxidative capacity. All results are reported in Table 2.

As can be seen from Table 2, the extract provided 50% inhibition at a concentration of 50 μg/mL; indicating lesser antioxidant capacity than positive controls, but it inhibited the superoxide radical more effectively than ascorbic acid. Hydroxyl radical scavenging and lipid peroxidation were not tested with ascorbic acid since this chemical was already present in the test medium. On the other hand, results in Table 2 demonstrated the strong ability of the essential oil to act as a donor for hydrogen atoms or electrons. The reduction of the stable radical DPPH to yellow coloured diphenylpicrylhydrazine was obtained with an IC_{50}=1.56 μg/mL instead of 3.90, 7.92, 19.30 μg/mL for ascorbic acid, curcumine, and BHT, respectively. Both hydroxyl radical scavenging and the lipid peroxidation inhibition of the essential oil were also better than curcumine and BHT. This could be attributed to the presence of some monoterpenes (Table 1). The antioxidative effectiveness in natural substances was reported to be mostly due to phenolic compounds (21). Phenolic compounds were reported to play an important role in inhibiting autoxidation of essential oils (22). In order to determine the antioxidant nature of the essential oil, its main components, e.g. eucalyptol, camphor, β-pinene, borneol, terpinen-4-ol, α-pinene were all tested individually and none exhibited antioxidative activity in all methods employed. Antioxidant activity of eucalyptol and terpinen-4-ol were previously reported using two different methods; aldehyde/carboxylic acid assay and lipid peroxidation (2) with prolonged incubation periods (30 days and 18 hr, respectively) in contrast to our experimental procedure employing a 30~60 min incubation period. These results suggest that the main components in the total essential oil might synergize with each other or with other components involved in these activities.

Antimicrobial activity

Water-insoluble portions of the methanolic extracts were found to have moderate activity against *Clostridium perfringens* and the yeasts. No activity was exhibited by the water-soluble portion. The water-insoluble portion exhibits, in many cases, greater activity than the water-soluble (aquatic) ones (23).

The essential oil possessed stronger antimicrobial activity than the extracts tested. The essential oil exhibited moderate activity against *Streptococcus pneumoniae*, *Clostridium perfringens* and *Candida albicans*, and weak activity against *Mycobacterium smegmatis*, *Acinetobacter lwoffii* and *Candida krusei* (Table 3). The growth inhibitions of test microorganisms ranged from 4.5 mg/mL (w/v) to 72.00 mg/mL (w/v) with the lowest MIC value against *Streptococcus pneumoniae*, *Clostridium perfringens*, *Candida albicans* at 4.5 mg/mL (w/v).

Eucalyptol (1,8-cineole) and camphor are well-known chemicals with pronounced antimicrobial potentials (16). Antimicrobial activities of borneol have also been previously reported by other investigators (24).

In conclusion, our observations confirm that essential oil of *Ledum palustre* L. possess strong antioxidative activity but low antimicrobial activity in vitro.

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Table 3. Antimicrobial activity of the essential oil and the methanolic extracts from *Ledum palustre* L.

Microorganism	Essential oil		MeOH[3]		The MIC of antibiotics[4]			
	DD[1]	MIC[2]	H_2O	$CHCl_3$	AK	CF	CM	FC
Staphylococcus aureus	8	72.00	na	na[5]	2.00	0.25	nt[6]	nt
Streptococcus pneumoniae	14	4.05	na	na	nt	nt	0.125	nt
Moraxella catarrhalis	na	na	na	na	nt	nt	nt	nt
Bacillus cereus	10	72.00	na	10	nt	nt	nt	nt
Acinetobacter lwoffii	15	18.00	na	na	nt	nt	nt	nt
Enterobacter aerogenes	7	72.00	na	na	nt	nt	nt	nt
Escherichia coli	na	na	na	na	2.00	0.015	nt	nt
Klebsiella pneumoniae	9	72.00	na	na	nt	nt	nt	nt
Proteus mirabilis	na	na	na	na	nt	nt	nt	nt
Pseudomonas aeruginosa	na	na	na	na	1.00	0.25	nt	nt
Clostridium perfringens	12	4.50	na	12	nt	nt	0.25	nt
Mycobacterium smegmatis	12	9.00	na	na	nt	nt	nt	nt
Candida albicans	21	4.50	na	12	nt	nt	nt	128.00
Candida krusei	16	18.00	na	12	nt	nt	nt	64.00

[1]DD, disc diffusion method as recommended by NCCLS. Diameter of zone of inhibition (mm) including disk diameter of 6 mm.
[2]MIC, minimum inhibitory concentration. Values given as mg/mL (for the essential oil) and as μg/mL (for antibiotics).
[3]MeOH, methanolic extracts. Diameter of zone of inhibition (mm) including well diameter of 8 mm.
[4]AK, amikacin; CF, ciprofloxacin; CF, clindamycin; FC, fluconazole.
[5]na, not active.
[6]nt, not tested.

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