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Antioxidant phenolics from *Lobaria pulmonaria* (L.) Hoffm. and *Usnea longissima* Ach. lichen species

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In this study, 1 steroidal and 8 phenolic compounds from *Lobaria pulmonaria* (L.) Hoffm. and *Usnea longissima* Ach. were isolated and their chemical structures were characterized by ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H COSY, HMQC, HMBC, IR, UV, and MS spectroscopic methods as stictic acid, isidiophorin, rhizonaldehyde, rhizonyl alcohol, pulmonarianin, vesuvianic acid, ergosterol peroxide, usnic acid, and diffractaic acid. Among these compounds, pulmonarianin, isolated from *L. pulmonaria*, was characterized for the first time as a natural compound. In addition to isolation and characterization, the lipid peroxidation inhibition and DPPH radical scavenging activities of the isolated compounds were investigated. Among these compounds, isidiophorin, rhizonaldehyde, rhizonyl alcohol, and pulmonarianin showed better lipid peroxidation inhibition in comparison to the other tested phenolics. However, usnic and diffractaic acids, which are phenolic compounds, did not have antioxidant potential.

Key Words: Antioxidants, lichens, *Lobaria pulmonaria*, *Usnea longissima*, pulmonarianin, rhizonaldehyde, rhizonyl alcohol

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Introduction

In living organisms, various reactive oxygen and nitrogen species can be formed by different mechanisms. Endogenous antioxidant molecules and systems can protect biomolecules against the harmful actions of reactive species. However, endogenous antioxidants may not be efficient in some cases; thus, exogenous antioxidant consumption may be important to organisms. Therefore, it is important to determine the antioxidant activities of plants and their metabolites to distinguish between endogenous and exogenous sources of antioxidants. For this purpose, we studied the antioxidant activities of the compounds isolated from 2 lichen species, *Lobaria pulmonaria* (L.) Hoffm. and *Usnea longissima* Ach.

Lichens are symbiotic organisms combining algal and fungal properties. The adaptability of such organisms to extreme environmental conditions, particularly to temperature-induced ones, is of much interest. *L. pulmonaria* has been widely used in folk remedies for the treatment of various diseases such as eczema, respiratory and pulmonary diseases, and arthritis; it is also used as food and in cosmetics. For example, *L. pulmonaria* has long been used as food, as a laxative cream, and for the treatment of respiratory disorders in Turkish folk medicine.[1] Antiulcerogenic and antiinflammatory properties of *L. pulmonaria* have been reported by our research group.[2] The lichen *Usnea longissima* has been used in the treatment of bone fractures and strains,[3] human leg injuries,[4] and ulcers.[5] Antioxidant and antiulcerogenic activities of *U. longissima* were recently reported by our research group.[6,7]

Phenolic compounds, including depsides, depsidones, dibenzofurans, and pulvinic acid derivatives, are secondary metabolites of lichen samples.[8] These compounds have attracted much attention in recent investigations because of their antiviral, antibiotic, antitumor, allergenic, and plant growth inhibitory activities.[8–10] Many depsides, depsidones, and dibenzofuran derivatives isolated from *U. longissima* and *L. pulmonaria* have also been reported.[9–11] Although the antioxidant activities of some depsides and depsidones isolated from several lichen species have recently been demonstrated,[12,13] the antioxidant properties of lichens are poorly known. There have also been limited reports on the relationship between the antioxidant activity of lichen samples and their pure phenolic compounds. Therefore, the aim of the present study was to isolate the phenolic compounds from 2 lichen species and to test for antioxidant properties.

Experimental

General experimental procedures

NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer, operating at 400 MHz and 100 MHz for ^{1}H-NMR and ^{13}C-NMR, respectively, using $CDCl_3$ and DMSO-d_6. Chemical shifts were expressed in δ (in ppm) downfield from TMS as an internal standard, and coupling constants were reported in Hz. The UV-visible spectra of the compounds and biochemical assays were recorded on Thermo Spectronic-HEλIOS β and Jasco V-530 spectrophotometers. The IR spectra were determined on a PerkinElmer Model 1600 FT-IR spectrophotometer. GC-MS was performed with a ThermoFinnigan Trace GC/Trace DSQ/A1300 (EI quadrupole) equipped with an SGE-BPX5 MS fused silica capillary column (30 m $\times$ 0.25 mm i.d., film thickness of 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. The carrier gas was helium at a flow rate of 1 mL min^{-1}. Injector and MS transfer line temperatures were set

at 220 °C and 290 °C, respectively. Melting points were determined with a Thermo Scientific 9200 apparatus. Optical rotation was measured on a Bellingham and Stanley ADP 220 polarimeter equipped with a sodium lamp and a 10-cm microcell with CH_2Cl_2 as the solvent at 20 °C. Column chromatography (CC) was carried out using silica gel 60 (70-230 and 230-400 mesh; Merck); thin layer chromatography (TLC) and PTLC were carried out on silica gel 60 precoated plates, F-254 (Merck). The spots on the TLC plates were visualized by UV_{254}, UV_{366}, and spraying with 1% vanillin-H_2SO_4 followed by heating (105 °C).

Lichen samples

Lobaria pulmonaria (L.) Hoffm. and *Usnea longissima* Ach. were collected from the Giresun region (northern Anatolia) of Turkey in June-September of 2007. They were identified by Dr. Ali Aslan and voucher specimens were deposited in the herbarium of Kazım Karabekir Education Faculty, Atatürk University, Erzurum, Turkey.

Extraction and isolation

A powdered sample (1 kg) of *L. pulmonaria* was extracted with acetone (3 L) at 50 °C under reflux conditions 5 times. After filtration, the extract was concentrated under vacuum using a rotary evaporator to yield 95 g of a brown residue. The concentrated extract (30 g) was fractioned on silica gel CC (400 g, 200-400 mesh) using $CHCl_3$-CH_3OH (9:1-6:4) as the eluent. The fractions (100 mL each) were compared by TLC (silica gel) using $CHCl_3$-CH_3OH (9:1 and 8:2), and the eluents giving similar spots in TLC were combined. In the end, 5 fractions (A-E) were obtained.

Fraction C afforded an amorphous, white precipitate, **1** (1.55 g), after elution. This fraction was determined to contain another major compound (**2**) besides compound **1**, monitored by TLC. This fraction (2.95 g) was further subjected to silica gel CC (250 g, 230-400 mesh) and eluted with $CHCl_3$-CH_3OH-H_2O (9.5:0.4:0.1), and thus **2** (770 mg) and **1** (830 mg) were isolated.

Fraction D (4.90 g) was subjected to silica gel CC (silica gel 60: 250 g, 200-400 mesh) and eluted with $CHCl_3$-MeOH (9.5:0.5 and 9:1), and thus compounds **3** (1.60 g) and **4** (925 mg) were isolated. Compound **4** (425 mg) was also isolated from Fraction E by spontaneous precipitation.

Fraction A (5.55 g) was further chromatographed on silica gel (200 g, 230-400 mesh) using $CHCl_3$ – *n*-hexane (7:3), and 4 fractions (AA-AD) were obtained according to the combination of the fractions that gave similar spots in TLC (silica gel). Fraction AD afforded an amorphous, white precipitate after elution. The precipitates were solved in $CHCl_3$ and controlled with TLC (silica gel) using Et_2O and $CHCl_3$:CH_3OH (9.5:0.5) solvent systems, and thus **5** (2.02 g) was obtained. Fraction AB (1.73 g) was subjected to silica gel CC (80 g, 230-400 mesh), eluting Et_2O:*n*-hexane (8:2), and a total of 81 fractions were obtained. Compound **6** (40 mg) was isolated from fractions 40-67 by preparative TLC (silica gel) using CH_2Cl_2:EtOAc (9:1). Fraction AC (2.82 g) was further separated by silica gel CC (120 g, 230-400 mesh) and eluted with $CHCl_3$:CH_3OH (9.75:0.75), and a total of 18 fractions were collected. Compound **7** (58 mg) was purified by PTLC (silica gel) using CH_2Cl_2:EtOAc (8:2) from combined fractions 10-18.

An air-dried sample of *Usnea longissima* (250 g) was extracted with 500 mL of diethyl ether using a Soxhlet apparatus at 40 °C. The crude extract of the lichen sample was filtered and stored at 4 °C for 24 h to precipitate usnic acid (UA). The UA precipitates were collected and subjected to silica gel CC (70-230 mesh)

by elution with a $CHCl_3$:*n*-hexane (8:2) solvent system. At the end of this process, 2.10 g of usnic acid (**8**) was obtained with a yield of 0.84% (w/w). After the usnic acid precipitates were removed, the solution was concentrated using an evaporator under reduced pressure. The extract (18.75 g) was subjected to CC using silica gel (70-230 mesh) and elution with $CHCl_3$:*n*-hexane (7:3, 7.5:2.5, 9:1, and 10:0) and $CHCl_3$:CH_3OH (9:1) solvent systems. Thus, 5.75 g of diffractaic acid (**9**) was purified.

Stictic acid (**1**): White needles; mp 270-272 °C (dec.); UV λ_{max} ($CHCl_3$) nm: 271, 296, 316, 330, 346; IR ν_{max} ($CHCl_3$) cm^{-1}: 3393 (-OH), 1740 (ester carbonyl), 1687, 1601, 1373, 1294, 1150, 1083. EIMS (70 eV) m/z (rel. int.): 386 $[M]^+$ (21), 314 (36), 286 (43), 258 (53), 230 (52), 193 (81), 191 (100), 188 (58), 149 (48), 83 (70). ^{1}H-NMR and ^{13}C-NMR: Table 1.

Table 1. ^{1}H- and ^{13}C-NMR data[a] (δ, ppm) of compounds **1** and **2** in $CDCl_3$.

1			**2**		
C	^{13}C (δ)	^{1}H (δ)	C	^{13}C (δ)	^{1}H (δ)
1	113.7	-	1	113.3	-
2	163.8	-	2	167.0	-
3	115.1	-	3	114.1	-
4	163.1	-	4	162.4	-
5	113.5	7.08, *s*	5	112.9	7.02, *s*
6	151.6	-	6	147.0	-
7	161.4	-	7	160.6	-
8	187.3	10.45, *s*	8	27.5	2.44, *s*
9	22.2	2.48, *s*	9	133.3	7.01, *d*, J = 16.8 Hz
1′	109.8	-	10	132.5	7.91, *d*, J = 16.8 Hz
2′	152.6	-	11	200.0	-
3′	121.4	-	12	121.4	2.40, *s*
4′	148.7	-	1′	109.8	-
5′	138.1	-	2′	152.6	-
6′	136.6	-	3′	121.4	-
7′	167.1	-	4′	148.8	-
8′	10.3	2.18, *s*	5′	138.4	-
9′	95.8	6.59, *d*, J = 8.1 Hz	6′	136.4	-
			7′	161.8	-
OCH_3	57.4	3.90, *s*	8′	10.3	2.17, *s*
OH	-	8.20, d, J = 8.1 Hz, 9′	9′	96.2	6.46, *s*
	-	10.19, b*s*, 2′			
			OCH_3	57.4	3.93, *s*
			OH	-	10.18, b*s*, 2′
					8.04, b*s*, 9′

[a] Assignments made by HMQC and ^{1}H-^{1}H COSY experiments.

Isidiophorin (**2**): White amorphous powder; mp 268-270 °C; UV λ_{max} ($CHCl_3$) nm: 265, 299, 307, 313, 354; IR ν_{max} ($CHCl_3$) cm^{-1}: 3390 (-OH), 1740 (ester carbonyl), 1602, 1430, 1377, 1289, 1146. EIMS (70 eV) m/z (rel. int.): 426 $[M]^+$ (2), 216 (15), 215 (32), 189 (31), 188 (100), 167 (17), 166 (20), 149 (41), 83 (19). ^{1}H-NMR and ^{13}C-NMR: Table 1.

Rhizonaldehyde (**3**): White amorphous powder; mp 248-249 °C (dec.); UV λ_{max} ($CHCl_3$) nm: 215, 265, 312; IR ν_{max} ($CHCl_3$) cm^{-1}: 3387 (-OH absorption), 1732 (aldehyde carbonyl), 1689, 1603, 1373, 1294, 1262, 1222, 1140, 1086. EIMS (70 eV) m/z (rel. int.): 181 $[M]^+$ (5), 164 (49), 163 (67), 135 (56), 134 (47), 121 (22), 79 (17), 67 (19), 44 (100), 40 (92). ^{1}H-NMR and ^{13}C-NMR: Table 2.

Table 2. ^{13}C- and ^{1}H-NMR data[a] (δ, ppm) of compounds **3** and **4** in DMSO-d_6.

	3		**4**	
Position	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)
1	113.8	-	113.5	-
2	161.5	-	138.4	-
3	115.1	-	119.1	-
4	163.1	-	162.4	-
5	113.4	7.07, *s*	112.1	6.90, *s*
6	151.5	-	144.6	-
7	187.4	10.42, *s*	51.9	4.53, *s*
8	10.2	2.17, *s*	10.1	2.12, *s*
9	22.2	2.48, *s*	21.6	2.41, *s*
OCH_3	57.4	3.89, *s*	56.9	3.83, *s*
O<u>H</u>	-	8.20, b*s*, 2		4.70, b*s*, 7 8.27, b*s*, 2

[a] Assignments made by HMQC and ^{1}H-^{1}H COSY experiments.

Rhizonyl alcohol (**4**): White amorphous powder; mp >250 °C (dec.); UV λ_{max} (DMSO) nm: 285, 304; IR ν_{max} ($CHCl_3$) cm^{-1}: 3435 (-OH), 2997, 2913, 1437, 1407, 1312, 1031. EIMS (70 eV) m/z (rel. int.): 182 $[M]^+$ (3), 166 (100), 165 (43), 148 (30), 120 (27), 106 (20), 91 (17), 77 (17), 135 (13). ^{1}H-NMR and ^{13}C-NMR: Table 2.

Pulmonarianin (**5**): White amorphous powder; $[\alpha]_D^{20} = -97°$; mp 246-248 °C (dec.); UV λ_{max} ($CHCl_3$) nm: 203, 246, 300, 334; IR ν_{max} ($CHCl_3$) cm^{-1}: 3425 (-OH), 2926 (aliphatic groups), 1751 (aldehyde carbonyl), 1734 (lactone carbonyl), 1691, 1604, 1553, 1434, 1401, 1369, 1295, 1211, 1130. EIMS (70 eV) m/z (rel. int.): 398 $[M]^+$ (8), 63 (100), 191 (86), 78 (76), 188 (9), 81 (74), 193 (59), 259 (51), 151 (48), 287 (40), 315 (30). ^{1}H-NMR and ^{13}C-NMR: Table 3.

Vesuvianic acid (**6**): White amorphous powder; mp 244-246 °C; UV λ_{max} ($CHCl_3$) nm: 203, 244, 310, 334; IR ν_{max} ($CHCl_3$) cm^{-1}: 3421 (-OH), 2927 (aliphatic groups), 1739 (aldehyde carbonyl), 1698 (lactone carbonyl), 1604, 1441, 1379, 1298, 1263, 1217. EIMS (70 eV) m/z (rel. int.): 414 $[M]^+$ (65), 370 (15), 369 (45),

368 (100), 341 (30), 340 (15), 312 (12), 287 (16), 285 (13), 210 (25), 191 (35), 58 (28), 43 (63). ^{1}H-NMR and ^{13}C-NMR: Table 3.

Table 3. ^{13}C- and ^{1}H-NMR data[a] (δ, ppm) of compounds **5** and **6** in $CDCl_3$.

	5		**6**		
C	^{13}C (δ)	^{1}H (δ)	C	^{13}C (δ)	^{1}H (δ)
1	115.7	-	1	115.5	-
2	161.5	-	2	164.5	-
3	114.8	-	3	115.0	-
4	165.0	-	4	163.9	-
5	111.9	6.76, *s*	5	112.2	6.75, *s*
6	151.5	-	6	151.8	-
7	161.0	-	7	161.3	-
8	187.4	10.37, *s*	8	187.0	10.54, *s*
9	22.4	2.55, *s*	9	22.6	2.57, *s*
1′	107.9	-	1′	108.5	-
2′	152.9	-	2′	152.8	-
3′	119.0	-	3′	121.8	-
4′	149.3	-	4′	149.7	-
5′	137.7	-	5′	138.0	-
6′	136.9	-	6′	132.6	-
7′	9.1	2.27, *s*	7′	170.0	-
8′a	47.0	2.82, *dd*, $J_1 = 17.9$ Hz, $J_2 = 9.3$ Hz	8′	9.4	2.30, *s*
8′b		3.88, *dd*, $J_1 = 17.9$ Hz, $J_2 = 1.8$ Hz	9′	102.4	6.49, *s*
9′	77.9	5.98, *dd*, $J_1 = 9.3$ Hz, $J_2 = 1.1$ Hz	1″	67.0	4.01, *q*, $J = 7.0$ Hz
10′	203.6	-	2″	15.2	1.28, *t*, $J = 7.0$ Hz
11′	30.1	2.33, *s*	OCH_3	56.9	3.98, *s*
OCH_3	56.8	3.97, *s*	O<u>H</u>	-	7.92, b*s*, 2′
O<u>H</u>	-	8.0, b*s*, 2′			

[a] Assignments made by HMQC and ^{1}H-^{1}H COSY experiments.

Ergosterol-5α,8α-peroxide (7): Colorless needles; mp 176-178 °C; UV λ_{max} ($CHCl_3$) nm: 202, 243, 305; IR ν_{max} ($CHCl_3$) cm^{-1}: 3423 (-OH), 2955 and 2895 (aliphatic groups), 1457, 1376, 1220. EIMS (70 eV) m/z (rel. int.): 428 $[M]^+$ (8), 55 (100), 69 (96), 43 (54), 83 (52), 85 (52), 93 (48), 67 (45), 95 (44), 107 (42), 41 (42), 91 (40), 301 (37), 109 (36), 133 (24), 145 (22), 267 (16), 330 (16). ^{1}H-NMR values (400 MHz, $CDCl_3$): δ 0.80 (*s*, H-18), 0.81 (*d*, $J = 6.6$ Hz, H-26), 0.81 (*d*, $J = 6.6$ Hz, H-27), 0.86 (*s*, H-19), 0.89 (*d*, 7.0 Hz, H-28), 0.98 (*d*, $J = 6.6$ Hz, H-21), 3.80-3.97 (*m*, H-3), 5.12 (*dd*, $J_1 = 15.4$ Hz, $J_2 = 8.05$ Hz, H-22), 5.20 (*dd*, $J_1 = 15.20$ Hz, $J_2 = 7.50$ Hz, H-23), 6.22 (*d*, $J = 8.6$ Hz, H-26), 6.48 (*d*, $J = 8.6$ Hz, H-7); ^{13}C-NMR values (100 MHz, $CDCl_3$): δ 13.1 (C-18), 17.8 (C-28), 18.4 (C-29), 19.9 (C-26), 20.2 (C-27), 20.8 (C-15), 21.1 (C-21), 23.6

(C-11), 28.8 (C-16), 30.3 (C-2), 33.3 (C-25), 34.9 (C-1), 37.1 (C-4), 37.2 (C-10), 39.6 (C-12), 39.9 (C-20), 43.0 (C-24), 44.8 (C-13), 51.3 (C-9), 51.9 (C-14), 56.4 (C-17), 66.6 (C-3), 79.6 (C-8), 82.4 (C-5), 130.9 (C-7), 132.5 (C-23), 135.4 (C-22), 135.7 (C-6).

Usnic acid (**8**): Yellow needles; mp 202-203 °C; IR ν_{max} ($CHCl_3$) cm^{-1}: 3423 (-OH), 2955 and 2895 (aliphatic groups), 1457, 1376, 1220; ^{1}H-NMR values (400 MHz, $CDCl_3$): δ 1.76 (s, H-13), 2.10 (s, H-16), 2.66 (s, H-15), 2.67 (s, H-18), 5.98 (s, H-4), 11.03 (s, 10-O<u>H</u>); ^{13}C-NMR values (100 MHz, $CDCl_3$): δ 9.5 (C-16), 29.8 (C-13), 33.2 (C-18), 34.1 (C-15), 61.1 (C-12), 100.3 (C-4), 103.5 (C-2), 105.9 (C-9), 107.2 (C-11), 111.3 (C-7), 157.2 (C-5), 159.5 (C-8), 165.9 (C-10), 181.3 (C-6), 193.7 (C-3), 200.0 (C-1), 202.3 (C-14), 203.7 (C-17).

Diffractaic acid (**9**): White amorphous powder; mp 189-190 °C; UV λ_{max} ($CHCl_3$) nm: 253, 315; IR ν_{max} (KBr) cm^{-1}: 3438 (-OH), 2927 (aliphatic groups), 1753 (carbonyl groups), 1651, 1625, 1597, 1472, 1446, 1395, 1319, 1293, 1242, 1165, 1089, 1063. EIMS (70 eV) m/z (rel. int.): 193 (100), 177 (0.9), 163 (1.8), 150 (6.3), 135 (1.8), 120 (0.9), 107 (2.2), 91 (3.5), 77 (2.6); ^{1}H-NMR values (400 MHz, $CDCl_3$): δ 2.17 (s, H-8), 2.19 (s, H-8′), 2.48 (s, H-9), 2.61 (s, H-9′), 3.86 and 3.87 (2x–OCH_3), 6.63 (s, H-5 and H-5′); ^{13}C-NMR values (100 MHz, $CDCl_3$): δ 9.1 (C-8), 9.4 (C-8′), 20.5 (C-9), 24.4 (C-9′), 55.9(-OC<u>H</u>$_3$), 62.4 (-OC<u>H</u>$_3$), 108.4 (C-5), 108.7 (C-3′), 117.0 (C-5′), 117.7 (C-3), 117.8 (C-1′), 119.7 (C-1), 135.8 (C-6), 141.4 (C-6′), 154.4 (C-4′), 157.4 (C-4), 160.3 (C-2), 164.1 (C-2′), 166.6 (C-7), 176.2 (C-7′).

Lipid peroxidation inhibition assays

The inhibitory effects of some isolated compounds on lipid peroxidation were evaluated with the lecithin liposome and the linoleic acid emulsion systems. Ferric chloride ($FeCl_3$) solution was added to the liposome and emulsion systems at a final concentration of 30 μM to increase the lipid peroxidation rate. Butylated hydroxytoluene (BHT) from Sigma was used as a reference antioxidant in the lipid peroxidation test systems. The final concentration of the compounds was 250 μg mL^{-1} in the lipid peroxidation incubation mixture. Stock solutions of the compounds were prepared with a chloroform and methanol mixture (9:1) and then slightly heated to increase the solubility.

Lecithin liposome system

The liposome test system was prepared according to previous reports.[14] The compounds and liposome solutions were added to a test tube, and then the solvent was evaporated under a stream of purified N_2 to form a thin film of lipid on the walls of the test tube. Each test tube contained 6 mg of lecithin and 500 μg of compound, and then 2 mL of phosphate buffer (0.05 M, pH 7.4) was added and the mixture was sonicated in a water bath sonicator for 15 min. Iron chloride ($FeCl_3$) solutions were added to produce a final concentration of 30 μM. Each sample was prepared in triplicate. The suspensions were capped and mixed well before incubation in an oven at 40 °C. At designated times, aliquots (0.4 mL) were transferred to microphage tubes and 0.8 mL of solution containing 0.375% (w/v) thiobarbituric acid (TBA) and 15% (w/v) trichloroacetic acid in 0.25 M HCl was added. The mixture was heated in a boiling water bath for 15 min, cooled, and centrifuged at 1500 rpm for 5 min. The absorbance of the supernatant liquid was measured at 533 nm with a Jasco V-530 spectrophotometer

against a blank prepared by adding 0.4 mL of phosphate buffer to 0.8 mL of TBA reagent and by heating in the same manner as for the samples.

Linoleic acid emulsion system

The effect of the compounds on the lipid peroxidation of linoleic acid emulsions was determined by the thiocyanate method.[15] The linoleic acid emulsion [0.393 g of linoleic acid (Sigma) + 0.393 g of Tween-20 (Sigma) in 70 mL of 0.067 M phosphate buffer, pH 7.4] was prepared by stirring at a high rate for 30 min. This linoleic acid emulsion (1.5 mL) was mixed with each isolated compound and $FeCl_3$ to give the desired final concentrations in the mixture. The final volume was adjusted to 2.0 mL with distilled water. Final phosphate buffer and iron concentrations were 0.05 M and 30 μM, respectively. Test tubes containing solutions were incubated in darkness at 40 °C. After designated intervals of incubation, lipid peroxides were determined by reading the absorbance at 508 nm after reaction with 50μ of the test mixture, 50μ of $FeCl_2$ (20 mM), and 50 μ of ammonium thiocyanate (30%) in 4.8 mL of methanol.

DPPH radical scavenging

This test was carried out as previously described.[15] Briefly, 0.25 mL of 0.5 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical solution (Fluka) in methanol was mixed with sample solutions and then the final volume was adjusted to 2 mL with methanol. The final extract concentration was 500 μg mL^{-1}. After 30 min of incubation in darkness, absorbance was measured at 517 nm. Decreased DPPH solution absorbance indicated increased DPPH radical scavenging activity. The control was the absorbance of the DPPH solution in the absence of added extracts. BHT was used as a known radical scavenging antioxidant for reference.

Results and discussion

The acetone extract of *L. pulmonaria* lichen species was subjected to fractionation by CC and TLC methods. In that process, 4 depsidones (**1**, **2**, **5**, and **6**), 2 rhizonic acid (4-*O*-methyl-β-orcinolcarboxyclic acid) derivatives (**3** and **4**), and 1 steroid (**7**) were isolated. The chemical structures of the compounds were determined by UV, IR, MS, and ^{1}H- and ^{13}C-NMR spectroscopic methods (Figure 1). The structures of the compounds were also supported by 1D-NMR (DEPT) and 2D-NMR methods (^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, and HMBC). From these data, it was concluded that compound **5** was a novel compound. Furthermore, isidiophorin (**2**), vesuvianic acid (**6**), and ergosterol-5α,8α-peroxide (**7**) were isolated from *L. pulmonaria* for the first time in this study. In addition, usnic acid (**8**) and diffractaic acid (**9**) were isolated from the ether extract of *U. longissima* to test their biological activities. The chemical structures of the known compounds were also confirmed by comparison of their spectral data with those reported in the literature, i.e. as stictic acid (**1**),[8] isidiophorin (**2**),[16,17] vesuvianic acid (**6**),[16] ergosterol-5α,8α-peroxide (**7**),[16,18] diffractaic acid,[16,19] and usnic acid,[16] and long-range ^{1}H-^{13}C correlations from their HMBC spectra. The NMR data of the known compounds, stictic acid (**1**) and isidiophorin (**2**), are given in Table 1. Isidiophorin (**2**) was previously characterized as a new compound from *L. isidiophora* Yoshim.[17] Furthermore, the NMR data of the other known compound, vesuvianic acid (**6**), are given in Table 3.

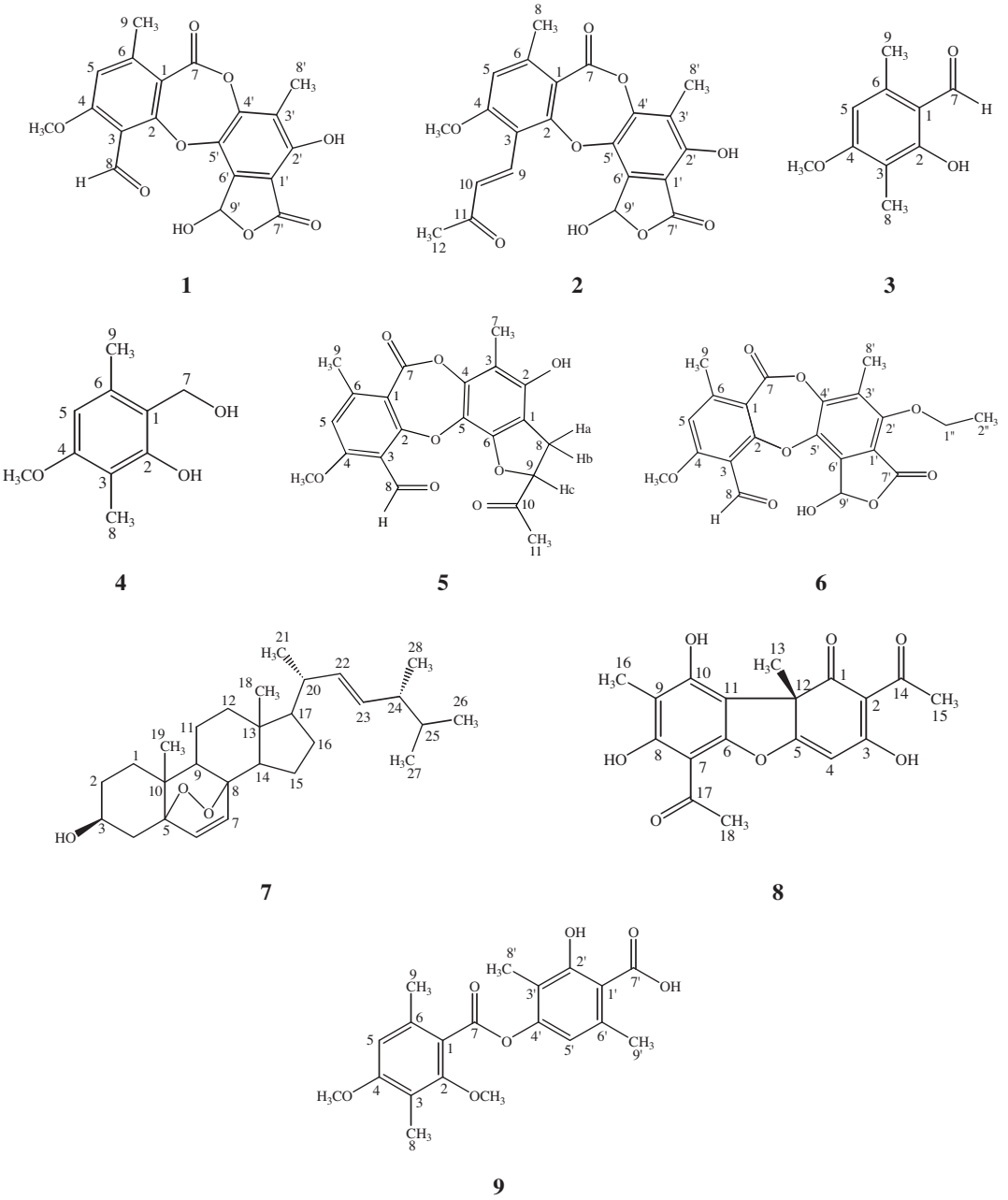


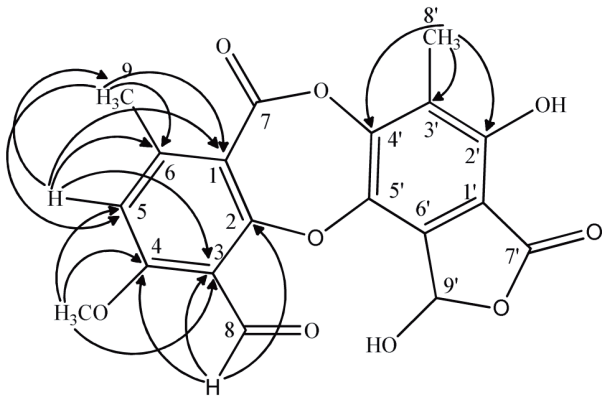
Figure 1. The chemical structures of compounds **1-9**.

The molecular formula of rhizonaldehyde (**3**) was deduced as $C_{10}H_{12}O_3$ by mass spectrum and ^{13}C-NMR spectra. In the ^{1}H-NMR spectrum of **3**, methyl singlets [δ 2.17 (H-8) and δ 2.47 (H-9)] were observed; the spectrum also contained a methoxyl group signal at δ 3.89. The ^{13}C-NMR and ^{1}H-NMR spectra of **3** showed the presence of aldehyde carbonyl groups [δ = 187.4 and δ = 10.42 (1H, *s*), respectively]. The aldehyde carbonyl absorption was observed at 1732 cm^{-1} in the IR spectrum of **3**. The ^{13}C-NMR spectrum of **3** revealed 6 olefinic carbon signals, confirming a benzene ring. In its DEPT ^{13}C-NMR spectra, 3 methyl

and 2 methine carbon signals were observed. In view of the ^{1}H-NMR, ^{13}C-NMR, IR, and UV data, the chemical structure of **3** was characterized as 4-methoxy-3,6-dimethyl-2-hydroxy benzaldehyde or 4-*O*-methyl-β-orcinolaldehyde. The correlations in the 2D ^{1}H-^{13}C heteronuclear COSY spectrum of **3** also allowed us to assign all protonated carbons and to confirm the structure. In the HMBC spectra of **3**, the correlations shown in Figure 2 confirm the proposed chemical structure of **3**. Previously, 4-hydroxy-2-methoxy-3,6-dimethyl benzaldehyde, which is an isomer of **3**, was reported from *Aspergillus silvaticus*,[16,20] and the spectral data were very similar to those of this compound. Thus, we thought that **3** might have been the same compound, 2-methoxy-3,6-dimethyl-4-hydroxybenzaldehyde. However, in the HMBC spectra of **3**, the correlation of δ_H 7.07 (H-5) with δ_C 163.1 (C-4) showed the methoxyl group linkage at C-4 (Figure 2) and that the chemical structure of **3** is 2-hydroxy-4-methoxy-3,6-dimethyl benzaldehyde (4-*O*-methyl-β-orcinolaldehyde or rhizonaldehyde). Although rhizonaldehyde was synthesized previously,[21−22] this compound was isolated from a natural source for the first time.

The molecular formula, $C_{10}H_{14}O_3$, of **4** was established by mass spectra on the molecular peak of 182 m/z and the ^{13}C-NMR spectrum. The NMR spectral data of **4** were very similar to those of **3**, except for the aldehyde proton and the aldehyde carbon signals in the ^{1}H-NMR and ^{13}C-NMR spectra, respectively, of **3** (Table 2). In the ^{1}H-NMR spectra of **4**, 2 methyl singlets [δ 2.12 (H-8) and δ 2.41 (H-9)] and 1 methoxyl singlet (δ 4.53) were observed. Furthermore, a signal at δ 4.53 in the proton spectrum and a signal δ 51.9 in the ^{13}C-NMR spectrum suggested that **4** contains a –C$\underline{H}_2$OH group. The fact that strong hydrogen bond absorption was observed at 3435 cm^{-1} in the IR spectrum of **4** confirmed that it is a benzyl alcohol derivative. The ^{13}C-NMR spectrum of **4** revealed 6 olefinic carbon signals, confirming a benzene ring. The DEPT ^{13}C-NMR spectrum of **4** showed that only one of these olefinic carbons signals is protonated, suggesting that the other olefinic carbons contain substituents. The correlations in the 2D ^{1}H-^{13}C heteronuclear COSY spectrum of **4** allowed us to assign all protonated carbons and also to confirm the structure. Based on the ^{1}H-NMR, ^{13}C-NMR, IR, MS, and UV data, the chemical structure of **4** was characterized as 2-(hydroxymethyl)-4-methoxy-3,6-dimethyl phenol, or rhizonyl alcohol. The HMBC spectrum of **4** is in accordance with the proposed chemical structure of **4** (Figure 2). As can be seen from Figure 2, the correlation of δ_H 6.90 (H-5) with δ_C 162.4 (C-4) in the HMBC spectra of **4** showed that the methoxyl group linkage was at C-4. Similar to rhizonaldehyde (**3**), rhizonyl alcohol (**4**) was isolated from *L. pulmonaria* as a natural lichen metabolite for the first time in the present study.

Compound **5** was obtained as colorless needles. The molecular formula of **5** was determined to be $C_{21}H_{18}O_8$ by the molecular ion peak at m/z 398 in the mass spectrum. Its IR spectrum displayed absorptions attributable to hydroxyl (3425 cm^{-1}) and carbonyl groups (1751 cm^{-1} and 1734 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectroscopic data of **5** were similar to those of stictic acid (**1**), with the exception of C-9 and C-7′ in stictic acid (**1**) instead of a methylene (δ 47.0), an oxygenated –CH carbon (δ 77.9), and a ketone carbonyl carbon (δ 203.6). In the ^{1}H-NMR spectrum of compound **5**, the H-8′ protons produced signals corresponding to an AB system (AB part, δ 2.82, *dd*, J_1 = 17.9 Hz, J_2 = 9.3 Hz; B part, δ 3.88, *dd*, J_1 = 17.9 Hz, J_2 = 1.8 Hz). The corresponding ^{1}H-NMR signals at δ 5.98 (*dd*, J_1 = 9.3 Hz, J_2 = 1.1 Hz) were assigned to H-9′. The correlations in the 2D ^{1}H-^{13}C heteronuclear COSY spectrum of **5** allowed us to assign all protonated carbons. In the HMQC spectrum of **5**, signals δ 2.27 (H-7′), δ 2.33 (H-11′), δ 2.55 (H-9), δ 2.82 and 3.88 (H-8′), δ 3.97 (-OC$\underline{H}_3$), δ 5.98 (H-9′), δ 6.76 (H-5), and δ 10.37 (H-8) correlated with δ 9.1 (C-7′), δ 30.1 (C-11′), δ 22.4 (C-9), δ 47.0 (C-8′), δ 56.8 (-O$\underline{C}H_3$), δ 77.9 (C-9′), δ 111.9 (C-5), and δ 187.4 (C-8), respectively.



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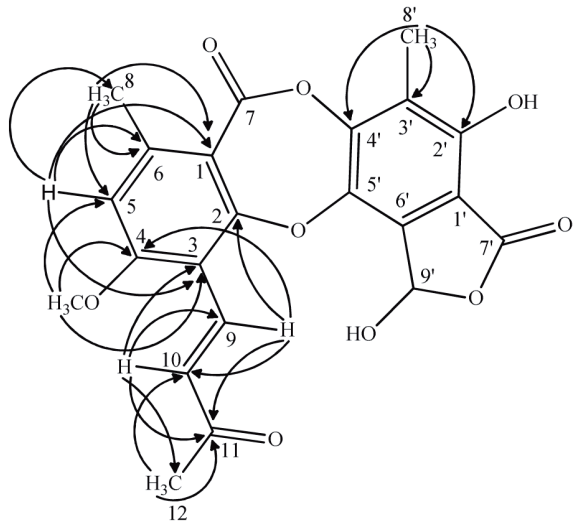

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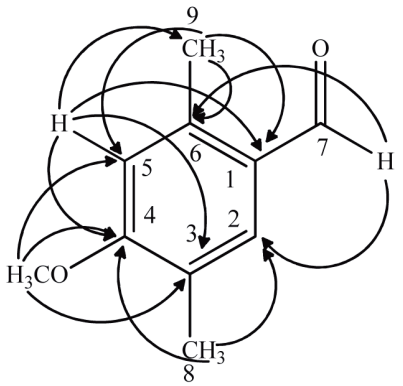

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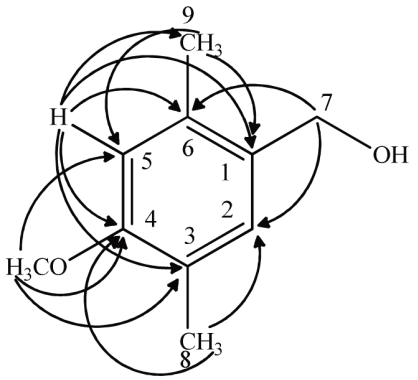

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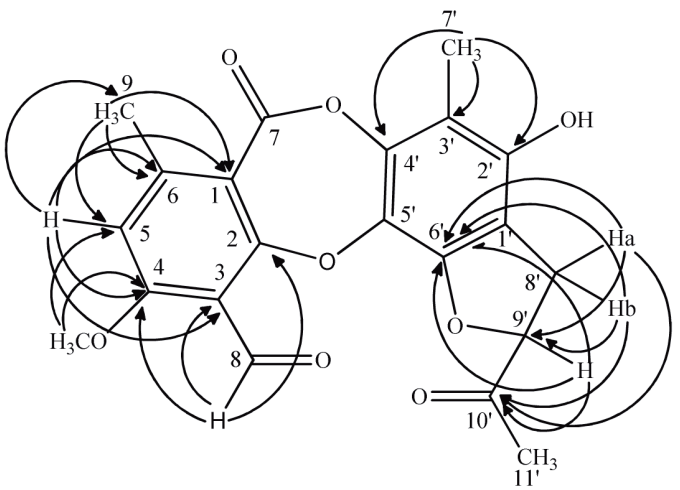

5

Figure 2. HMBC correlations of compounds **1-5**.

HMBC and ^{1}H-^{1}H COSY spectra also confirmed this deduction. In the HMBC spectra of **5**, correlations of the H-8′ protons with C-6′, C-9′, and C-10′, and the J values observed for H-8′a, H-8′b, and H-9 in ^{1}H-NMR spectra, also confirmed the proposed structure of **5**. As shown in Figure 2, all HMBC correlations corroborated the proposed structure of **5**. According to our literature survey, compound **5** has not been previously identified and thus it was named pulmonarianin.

Two lipid peroxidation test systems (liposome and emulsion systems) were used for determining the lipid peroxidation inhibition potential of the isolated compounds. The peroxidation of lipids was spectrophotometrically monitored at intervals. BHT was used as the positive control in all tests. As can be seen in Figures 3 and 4, BHT was a strong antioxidant in both test systems. On the other hand, isidiophorin, rhizonaldehyde (**3**), rhizonyl alcohol (**4**), and pulmonarianin (**5**) retarded lipid peroxidation at different levels in both test systems. However, stictic acid and ergosterol peroxide exhibited antioxidant activity in only the liposome test system. Usnic acid (**8**) and diffractaic acid (**9**) were not antioxidants in either system; these compounds acted as prooxidants in the emulsion system, because absorbance of usnic acid is higher than that of the control in the first 2 data sets, which were obtained after 6 and 24 h of incubation. Stictic acid was also a prooxidant in the emulsion system.

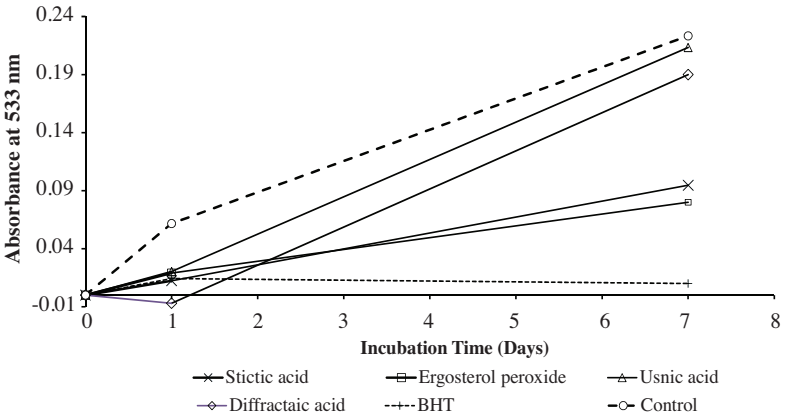

Figure 3a. Effects of the compounds and BHT (250 μg mL^{-1}) on lipid peroxidation in the lecithin liposome test system.

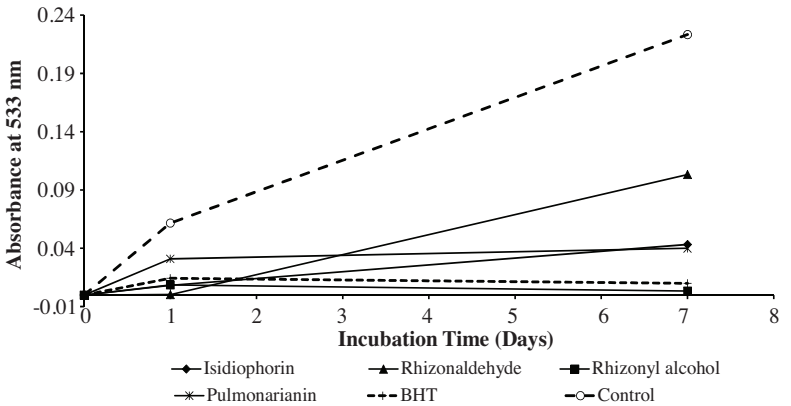

Figure 3b. Effects of the compounds and BHT (250 μg mL^{-1}) on lipid peroxidation in the lecithin liposome test system.

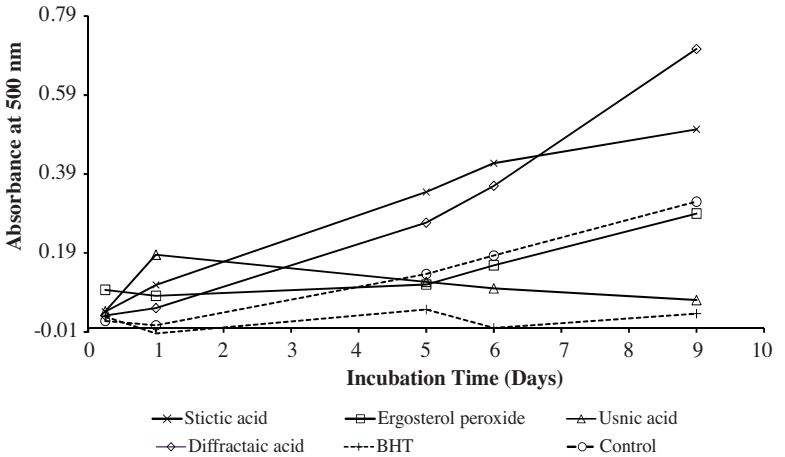

Figure 4a. Effects of the compounds and BHT (250 μg mL^{-1}) on lipid peroxidation in the linoleic acid emulsion test system.

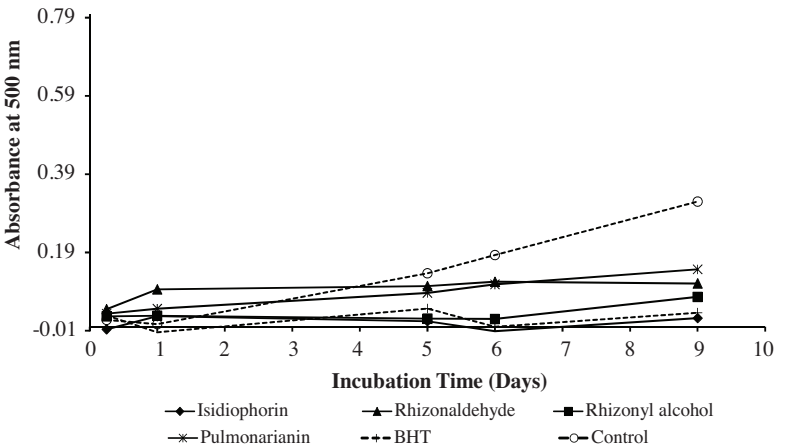

Figure 4b. Effects of the compounds and BHT (250 μg mL^{-1}) on lipid peroxidation in the linoleic acid emulsion test system.

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activities of the lichen metabolites were determined, except for vesuvianic acid, which could not be determined because of the low amount of the compound that was isolated. Low absorbance indicated high levels of DPPH scavenging activity. All compounds, except for usnic and diffractaic acids, showed DPPH radical scavenging activity (Figure 5). As can be seen in Figure 5, isidiophorin (**2**), rhizonaldehyde (**3**), rhizonyl alcohol (**4**), and pulmonarianin (**5**), which inhibited lipid peroxidation in both test systems, were DPPH radical scavengers. However, their DPPH scavenging activities, except for that of isidiophorin, were not high compared with that of BHT. Interestingly, ergosterol peroxide (**7**) acted as a lipid peroxidation inhibitor in the liposome test system and as radical scavenger, whereas it was not a lipid peroxidation inhibitor in the emulsion test system.

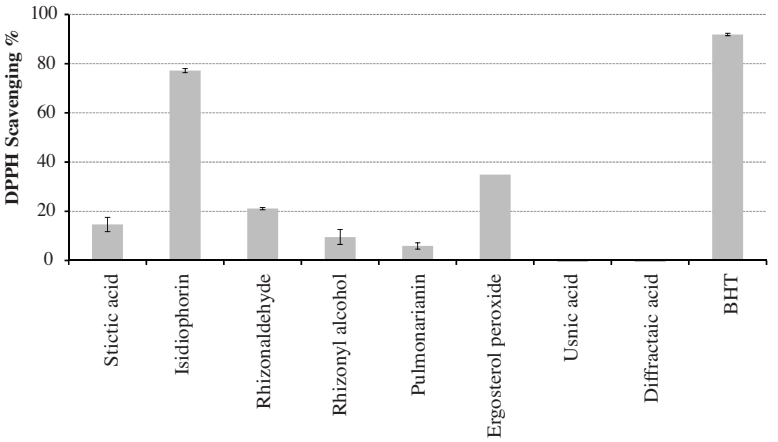

Figure 5. DPPH radical scavenging activities of the compounds and BHT (500 μg mL^{-1}).

It is well known that many phenolic compounds in the plant kingdom have antioxidant activity. In the current study, 1 steroidal and 8 phenolic compounds were isolated from lichens. Interestingly, usnic acid (**8**) and diffractaic acid (**9**), which are phenolic compounds, did not inhibit lipid peroxidation in either test system and did not scavenge the DPPH radical. It has been previously reported that these compounds cannot scavenge DPPH radicals, in accordance with our results.[23] On the other hand, the other phenolic compounds exhibited varying degrees of antioxidant activity, inhibiting lipid peroxidation in at least one of the test systems. Therefore, our results show that not all phenolic compounds have antioxidant potential. It has also been shown that depsidones have better antioxidant activity than depsides or usnic acid.[12,13,24,25] Similarly, in the current study, depsidones [stictic acid (**1**), isidiophorin (**2**), and pulmonarianin (**5**)] were better antioxidants, especially in the liposome system, when compared with usnic acid (**7**) and diffractaic acid (**8**).

Stictic acid (**1**) and isidiophorin (**2**) have similar structures. Isidiophorin has an α,β-unsaturated carbonyl group instead of the aldehyde group in the C-3 position of stictic acid (**1**). However, these small structural differences cause dramatically large changes in the DPPH scavenging and lipid peroxidation inhibition activities of isidiophorin. Hence, the α,β-unsaturated carbonyl group appears to be responsible for these changes in the antioxidant activities.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

1. Öztürk, S. *Bilim ve Teknik* **1995**, *328*, 74-77.
2. Süleyman, H.; Odabasoglu, F.; Aslan, A.; Cakir, A.; Karagoz, Y.; Gocer, F.; Halici, M.; Bayir, Y. *Phytomedicine* **2003**, *10*, 552-557.
3. Brij, L.; Upreti, D. K. *Lichenologist* **1995**, 27, 77-79.

4. Turner, N. J. *Ethnobotany of the Nitinaht Indians of Vancouver Island*, Government of Canada, British Columbia, 1983.
5. Chopra, R. N.; Chopra, I. C.; Handa, K. L.; Kapur, L. D. *Chopra's Indigenous Drugs of India*, U. N. Dhur & Sons Ltd., Calcutta, 1958.
6. Odabasoglu, F.; Aslan, A.; Cakir, A.; Suleyman, H.; Karagoz, Y.; Halici, M.; Bayir, Y. *Phytotherapy Res.* **2004**, *18*, 938-941.
7. Halici, M., Odabasoglu, F.; Suleyman, H.; Cakir, A.; Aslan, A.; Bayir, Y. *Phytomedicine* **2005**, *12*, 656-662.
8. Huneck, S. *Naturwissenschaften* **1999**, *86*, 559-570.
9. Nishitoba, Y.; Nishimura, I.; Nishiyama, T.; Mizutani, J. *Phytochemistry* **1987**, *26*, 3181-3185.
10. Honda, N. K.; Vilegas, W. *Quimica Nova* **1999**, *22*, 110-125.
11. Yamamoto, Y.; Miura, Y.; Kinoshita, Y.; Higuchi, M.; Yamada, Y.; Murakami, A.; Ohigashi, H.; Koshimizu, K. *Chem. Pharm. Bull.* **1995**, *43*, 1388-1390.
12. Hidalgo, M. E.; Fernandez, E.; Quilhot, W.; Lissi, E. *Phytochemistry* **1994**, *37*, 1585-1587.
13. Jayaprakasha, G. K.; Rao, L. J. *Zeitsch. für Naturf.* **2000**, *55*, 1018-1022.
14. Lawrence, G. D.; Mavi, A.; Meral, K. *Carbohyd. Res.* **2008**, *343*, 626-635.
15. Unal, E. L.; Mavi, A.; Kara, A. A.; Cakir, A.; Sengul, M.; Yildirim, A. *Pharm. Biol.* **2008**, *46*, 207-224.
16. Huneck, S.; Yoshimura, I. *Identification of Lichen Substances*, Springer Verlag, Berlin, 1996.
17. Bo, L., Zhongen, L.; Handong, S. *Act. Bot. Yunn.* **1990**, *12*, 447-448.
18. Hybelbauerova, S.; Sejbal, J.; Dracinsky, M.; Hahnova, A.; Koutek, B. *Chem. Biodivers.* **2008**, *5*, 743-750.
19. Bayir, Y.; Odabasoglu, F.; Cakir, A.; Aslan, A.; Suleyman, H.; Halici, M.; Kazaz, C. *Phytomedicine* **2006**, *13*, 584-590.
20. Kawahara, N.; Nozawa, K.; Nakajima, S.; Udagawa, S.; Kawai, K. *Chem. Pharm. Bull.* **1988**, *36*, 398-400.
21. Shah, R. C.; Samant, B .V. *P. Math. Sci.* **1938**, *7*, 266-268.
22. Robertson, A.; Stephenson, R. J. *J. Chem. Soc.* **1930**, 313-320.
23. Kumar, S.; Muller, K. *J. Nat. Prod.* **1999**, *62*, 817-820.
24. Devehat, F. L. L.; Tomasi, S.; Elix, J. A.; Bernard, A.; Rouaud, I.; Uriac, P.; Boustie, J. *J. Nat. Prod.* **2007**, *70*, 1218-1220.
25. Lopes, T. I. B.; Coelho, R. G.; Yoshida, N. C.; Honda, N. K. *Chem. Pharm. Bull.* **2008**, *56*, 1551-1554.