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BIODIVERSITY OF THE CHEMICAL CONSTITUENTS IN THE EPIPHYTIC LICHENIZED ASCOMYCETE *RAMALINA LACERA* GROWN ON DIFFERENCE SUBSTRATES *CRATAEGUS SINAICUS*, *PINUS HALEPENSIS,* AND *QUERCUS CALLIPRINOS*

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Aim: The identification and evaluation of lichen metabolite production by the epiphytic lichenized ascomycete *Ramalina lacera* collected from different substrates: *Crataegus sinaicus*, *Pinus halepensis*, and *Quercus calliprinos*.

Methods: Chemical constituents were characterized by GC-MS, HPLC, HR-TLC, and other chemical methods.

Results: The most abundant fatty acids were α-linolenic acid, oleic acid, and palmitic acid but a considerable variability of the ester composition from one to another was found. A comparison of neutral lipids, glycolipids, polar lipids and fatty acid composition of the tree-growing lichen *Ramalina lacera* was done. Diacylglyceryl-N,N,N-trimethylhomoserine, diaclyglycerylhydroxymethyl-N,N,N-trimethyl-β-alanine, phosphatidylcholine, and phosphatidylinositol were found as major components among polar lipids. Diffractaic, lecanoric, norstictic, protocetric, and usnic acids were isolated as major aromatic compounds in all samples of *R. lacera*.

Conclusions: We evaluated a diversity of fatty acids, lipids, and aromatic compounds produced by the samples of *Ramalina lacera* growing on different tree-substrates, *Crataegus sinaicus*, *Pinus halepensis* and *Quercus calliprinos*.

INTRODUCTION

Lichens are symbiotic associations between certain types of fungi (usually ascomycetes) and various green algae or cyanobacteria[1, 2]. Lichens have been used for a number of years as natural bioindicators for various heavy metals and as sources of information for environmental monitoring[3, 4]. These organisms have both algal and fungal properties and produce *n*-alkanes[5], unusual betaine ether glycerols[6], glyco- and phospholipids[7, 8], and saturated, unsaturated, branched[8, 9], and halogenated fatty acids[10]. Many different bioactive secondary metabolites have also been isolated from lichen species[11–13] which have been used in pharmaceutical and biotechnological sciences and industry[14–16].

Some *Ramalina* species are usually used as food in some Central and South Eastern Asian countries. Thus, lichens are used as traditional food by Rai and Limbu communities of East Nepal. Lichens *Ramalina farinacea, R. conduplicans, R. sinensis*, and *R. subfarinacea* are cooked mixed with various foods[17]. *Ramalina farinacea* and *R. conduplicans* are usually used as traditional food by Rai and Limbu communities of East Nepal. Lichens are cooked mixed with various foods[17]. Since the beginning of the 20th century hair powder of *Ramalina calicaris*, and *Ramalina* spp. Have been used in cosmetics in Europe[18], and India[19] respectively. Extracts from the lichen *Ramalina farinacea* were evaluated against fifteen clinical isolates of *Staphylococcus aureus*[20]. The aqueous extract of the *Ramalina farinacea* has folkloric reputation for the treatment of mental disorders in Africa; and tinctures have also been used for treatment of ringworm tinea in Nigeria[21]. In southwestern province Yunnan (China), the Yi, Dai, and Han ethnic peoples cook these two species of *Ramalina* (*R. conduplicans* and *R.sinensis*) to prepare a traditional cold dish served at marriage banquets[22].

The lichen substances are unique as they are unknown in other plant sources. Lichens contain many characteristic aromatic compounds with known antiviral, antimicrobial, antiproliferative, antimitotic, antioxidant activities[23]. Lichens may be a good potential source of bioactive phytochemicals[7, 13, 24].

In this study, we evaluated which fatty acids, lipids, and aromatic compounds are produced by the samples of *Ramalina lacera* growing on different tree-substrates, *Crataegus sinaicus*, *Pinus halepensis* and *Quercus calliprinos*.

MATERIALS AND METHODS

Plant material

Ramalina lacera (With.) J.R. Laundon (family Ramalinaceae) is a fruticose lichen growing on *Crataegus si-*

Table 1. Fatty acid composition of the *Ramalina lacera* growing on different substrates

Fatty acids	Substrates		
	Crataegus sinaicus (sample 1)	*Pinus halepensis* (sample 2)	*Quercus calliprinos* (sample 3)
Saturated	**26.00**	**29.38**	**18.36**
12:0	1.26	0.98	0.56
13:0	0.54	0.67	0.51
14:0	1.87	1.96	2.14
15:0	0.54	0.62	0.71
iso-15:0	0.81	0.72	0.63
anteiso-15:0	0.62	0.63	0.51
16:0	12.94	11.88	8.47
iso-17:0	0.53	0.54	>0.5
anteiso-17:0	0.62	>0.5	>0.5
18:0	5.13	4.96	3.29
20:0	1.14	0.97	0.56
Monoenes	**17.63**	**15.39**	**17.73**
15:1(*n*-8)	0.88	0.72	0.76
16:1(*n*-9)	5.14	2.15	4.96
16:1(*n*-7)	1.73	2.22	1.86
18:1(*n*-11)	0.67	0.74	0.56
18:1(*n*-9)	6.98	7.23	8.14
18:1(*n*-7)	1.58	1.60	0.94
20:1(*n*-9)	0.65	0.73	0.51
Dienes	**7.21**	**7.39**	**9.62**
16:2(*n*-4)	0.72	0.62	0.55
18:2(*n*-6)	5.94	6.16	8.57
20:2(*n*-6)	0.55	0.61	0.50
Polyenes	**49.16**	**47.84**	**54.28**
16:3(*n*-6)	1.76	2.22	2.77
16:3(*n*-3)	2.98	2.87	3.11
18:3(*n*-6)	2.14	2.67	3.04
18:3(*n*-3)	35.87	30.19	29.70
16:4(*n*-3)	3.14	2.98	3.49
18:4(*n*-3)	1.85	1.70	2.98
20:4(*n*-6)	0.96	1.21	2.25
20:4(*n*-3)	1.34	2.11	3.66
20:5(*n*-3)	1.12	1.89	3.28

naicus (family Rosaceae, voucher specimen HAI-0-30521 (MT), *Pinus halepensis* (family Pinaceae, voucher specimen HAI-0-30522 (MT), and *Quercus calliprinos* (family Fagaceae, voucher specimen HAI-0-30523 (MT), in the forests on Mount Carmel at 800 meters above sea level. All samples were collected in July 2003 from Mount Carmel, Sekher Pool (North Israel), identified and voucher specimen HAI-0-30521-305023 (MT) are deposited in the lichen herbarium of Biodiversity and Biotechnology Center of Cryptogamic Plants and Fungi (Haifa).

General extraction procedures

Clearly fresh lichen (50-75 g of each sample) was extracted (Soxhlet) with ethanol-water-HCl (90:10:1, v/v; 60 °C) over 72 h (fraction 1). The ethanolic residue was further extracted by light petroleum (60–80 °C, fraction 2), and then dichloromethane (fraction 3). Fraction 1 was concentrated *in vacuum* at 35 °C, water layer was lyophilized, and then dissolved in 2 ml of ethanol. Fraction 2 and 3 were separately concentrated to dryness *in vacuum* at 5 °C under reduced pressure, and then dissolved in 2 ml of a cold mixture ethanol-dichloromethane (1:1, v/v), which was used for separation by HPLC, TLC, and followed chemical analysis.

Gas chromatographic-mass spectrometric analysis

A Hewlett Packard 6890 (series II) gas chromatograph modified for glass-capillary work and a HP-GC mass selective detector (5973B MSD) were used. Fatty acid methyl esters were prepared and analyzed by GC fitted with serially coupled capillary columns: the RTX 1 column (30 m, ID 0.32 mm, film thick-ness 0.25 μm; Restek, USA) was coupled with a second capillary column (RTX 1701, 30 m, 0.32 mm, 0.25 μm film; Restek, USA). The instrumental

Table 2. Lipid composition of the *Ramalina lacera* growing on different substrates

Lipid classes	Substrates		
	Crataegus sinaicus (sample 1)	*Pinus halepensis (sample 2)*	*Quercus calliprinos (sample 3)*
Total lipids (mg/g dry wt)	36.9	42.4	51.3
Neutral lipids (mg/g dry wt)	22.1	26.8	32.1
Free fatty acids[#]	2.6 ± 0.2	1.6 ± 0.1	2.9 ± 0.4
Free sterols	3.9 ± 0.4	6.6 ± 0.3	4.6 ± 0.2
Diacylglycerols	1.5 ± 0.1	2.3 ± 0.2	3.1 ± 0.3
Triacylglycerols	10.1 ± 0.6	8.5 ± 0.7	12.3 ± 0.9
Steryl esters	2.9 ± 0.2	4.2 ± 0.2	6.2 ± 0.5
Wax esters	1.6 ± 0.1	3.2 ± 0.3	3.0 ± 0.6
Glycolipids (mg/g dry wt)	9.0	8.0	11.3
MGDG	3.2 ± 0.6	2.6 ± 0.4	3.2 ± 0.3
DGDG	5.0 ± 0.8	4.7 ± 0.6	7.2 ± 0.5
SQDG	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1
Polar lipids (% of total polar lipids)	5.8	7.6	7.9
DGTA	14.2 ± 0.8	12.6 ± 0.9	8.9 ± 0.6
DGTS	18.6 ± 0.9	21.3 ± 1.3	25.2 ± 1.9
PC	35.6 ± 1.4	44.2 ± 3.7	34.9 ± 2.8
PE	6.8 ± 0.4	7.4 ± 0.6	14.3 ± 0.9
PI	15.3 ± 0.7	11.2 ± 0.8	16.7 ± 0.7
PA	2.2 ± 0.2	0.9 ± 0.1	
X	7.3 ± 0.4	2.4 ± 0.2	

[#] Mean ± standard deviation.
Abbreviations: MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol, DGTA, diacylglyceryltrimethylalanine; DGTS, diacylglyceryltrimethylhomoserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; phosphatidic acid; X, non-identified polar lipid

Table 3. Identified aromatic compounds of the *Ramalina lacera* growing on different substrates

Aromatic Compounds (μg/100 g dry wt)	Substrates		
	Crataegus sinaicus (sample 1)	*Pinus halepensis (sample 2)*	*Quercus calliprinos (sample 3)*
Orsellinic acid	11.4	12.5	19.2
Lecanoric acid	22.5	26.2	21.7
Protocetric acid	27.4	32.8	41.2
Diffractaic acid	28.7	33.7	38.9
Homosekikaic acid	4.9	5.9	6.2
Usnic acid	26.8	25.3	29.4
Norstictic acid	23.5	29.9	22.8
Total	**145.2**	**166.3**	**179.4**

settings used were as follows: initial temperature, 40°C; initial time, 2.00 min; rate, 2 °C/min; final temperature, 300 °C, final time, 20 min; injection port, 180 °C; carrier gas, He: flow rate, 25.0 mL/min. The MS detector operated at 194 °C; ionization energy, 70 eV. The scan range, 30 to 700 *m/z* at 0.9 scan per sec. Solvent delay, 9 min. Fatty acid methyl esters were identified using mass spectral libraries search (Wiley 7th, and NIST-98).

High-Performance Liquid Chromatographic Analysis

The dried samples from fractions were reconstituted in 200 μL of methanol and analyzed using a Hewlett Packard 1100 HPLC system (Hewlett-Packard 1100 HPLC System w/ UV/VIS detector, includes: G1311A Quaternary Pump, G1314A UV/Vis Detector, G1313A Autosampler, G1322A Vacuum Degasser, Solvent Module, HP Chemstation with Computer System) with a photo diode array detec-

tor set at a range of 200–450 nm; all peaks were analyzed at 254 nm. An analytical reverse phase C_{18} column (A Spherisorb 5 ODS 2 column 250 × 4.6 mm, 5 μm; (Kontron) was used as the stationary phase. Mobile phase A contained 10 % methanol and 90 % water brought to a pH of 2.0 with phosphoric acid, and mobile phase B was 100% methanol. A linear gradient was applied over 30 min starting with 100% of mobile phase A at the start to 100% mobile phase B at the end. Chromatograms were analyzed by Hewlett Packard software; retention time and absorbance spectra were used to identify compounds, and also pure compounds were used for spectral analysis. Orsellinic and usnic acids were obtained from Sigma-IL, and used also as standard compounds. Isolated metabolites were identified with ^{13}C-NMR, IR, UV, and chemical methods as described previously[11].

High-Performance Thin-Layer Chromatographic Analysis

Total lipids were separated by column chromatography to neutral, glycolipid, and polar lipid fractions on silica gel (Merck 63/200 mesh). The obtained fractions were further analyzed by HR-TLC as described previously[25, 26]. Neutral lipids were separated on 10 × 20 cm silica gel plates (Silica Gel 60, Merck) with toluene-hexane-formic acid (150:70:2, v/v) mixture. Glycolipids were separated using acetone-benzene-water (100:40:9, v/v) mixture as described[27, 28]. Polar lipids, including betaine lipids and phospholipids, were separated with the help of chloroform-acetone-methanol-formic acid-water (150:20:10:10:4, v/v) mixture in the first direction, and acetone-benzene-formic acid-water (200:30:4:10, v/v) one in the second direction as described previously[29].

RESULT AND DISCUSSION

Ramalina lacera is a moderately xeric epiphytic fruticose lichen that grows in the Mediterranean areas on different shrubs and trees. These epiphytic species belonging to the family Ramalinaceae were chosen for a comparative examination of their fatty acid, polar lipids, and aromatic compounds. The GC-MS analysis of fatty acids in *R. lacera*, which grows on *Crataegus sinaicus*, *Pinus halepensis*, and *Quercus calliprinos*, revealed a high polyenoic content in species which grows on the Palestine oak *Q. calliprinos* (54.28 %, Table l), but much lower amounts of such acids in two other ones, viz. 49.16 % and 47.84 %, respectively. The amounts of trienoic acids, however, were higher in the samples from tree-growing species, 42.75 % (sample 1), 37.95 % (sample 2) and 38.62 % (sample 3). A total of 11 saturated fatty acids were identified, with *n*-16:0 and *n*-18:0 as major ones (8.47-12.94 % and 3.29-5.13 %, respectively). Total saturation in various species varied from 18.36 (sample 3) to 26 % (sample 1). All three species studied had almost identical monoenoic and dienoic acid contents. Among other interesting acids, 16:4(n-3), the presence of which is characteristic for green marine algae[27], and 18:4(n-3), characteristic for brown marine algae[28], were detected. It seems likely that the lichen photobiont synthesize these acids[5]. In sample 3, the total level of isomers of 20:4(n-6) and 20:4(n-3) was considerably higher than that from the rest of the examined species, reaching 5.91 %. These amounts of arachidonic acid isomers are the highest known in the lichen literature with regard to the total lipid extract, although still higher levels have been found in individual lipid classes, for example, *Peltigera aphthosa*[8].

Total lipid content was studied in all collected lichen species, having common climatic peculiarities. Table 2 shows total lipid compositions in lichens collected during July; total lipid content in such lichens show variations from 36.9-51.3 mg/g dry wt. Neutral lipids make up the highest percentage among of total lipids (Table 2) and vary from 22.1-32.1 mg/g dry wt. Examination of neutral lipids using HR-TLC revealed the domination of TAG and diacylglycerols over the rest of neutral lipids thereby representing more than 50% in the majority of lichen species. Free fatty acids, free sterols and its esters were also detected. The amount of glycolipid is comparatively lower than that of neutral lipids and varies between 8-11 %.

Examination of the lichen polar lipids, including betaine lipids (DGTA and DGTS) and phospholipids showed phosphatidylcholine (PC) to be the major phospholipids with concentrations varying from 34.9 to 44.2 % of total polar lipids (Table 2). The PC content in various fungal species is known to vary from 20 to 55%, whereas PC contained in red algal species varies from 61.6 to 77.8 % (ref. [23]). Phosphatidylethanolamine (PE) was detected in all lichen species studied; its level was low in the first two species (ca 6.8 %), but reached 14.3 % in sample 3. Phospatidylinositol was also found in all species studied; its level was highest in sample 3 (16.7 %). Both betaine lipids were detected in all samples; their level varies from 8.9 to 14.2 % for DGTA, and 18.6–25.2 % for DGTS.

DGTS, one of the three known betaine lipids, has been the object of many studies[24]. Betaine lipids occur in bacteria[30], fungi[31, 32], moss species[33], and in a number of brown, green and red algae[26, 29, 34] as well as in lichens[7, 35, 36]. As for higher plants, betaine lipids have been found in bryophytes[31, 37], in ferns[38], and other plants[39]. DGTA was detected in fungi[24], marine brown algae[27], and microalgal species[34].

HPLC has become more widely used as an effective analytical tool for the separation and identification of lichen substances[40]. Feige and co-workers[41] used HPLC with reversed-phase columns and gradient elution for separation of 331 lichen compounds. We used HPLC for separation aromatic compounds from three lichen samples. Seven aromaric acids were identified (Table 3). Total aromatic compounds varies from 145.2 (sample 1) to 179.4 μg/100 g dry wt (sample 3). Examination of the lichen aromatics showed lecanoric, protocetric, diffractaic, usnic, and norstictic acids to be the major metabolites (Table 3).

Earlier, aromatic compounds were isolated from the genus *Ramalina*. Thus, norstictic and salazinic acids

were isolated from *Ramalina subfarinacea*[42], and from *Ramalina farinacea* (Hawaii)[43]; usnic and sekikaic acids were isolated from Indian *R. tayloriana* growing on sandal trees[44]; and orsellic, lecanoric, diffractaic, protocetraric, usnic and norstictic acids were isolated from *R. lacera*[45]. Usnic acid was detected in *Ramalina yasudae*[46]. Trivaric and divaric acids are the major products of the *Ramalina americana*, and lecanoric and gyrophoric acids were identified as minor metabolites[47]. Chilean native *Ramalina* species: *R.chilensis*, and *R. farinacea* contains lecanoric, divaricatic, salazinic, usnic, norstictic, and ramalinolic acids[48, 49]. New Zealand's *Ramalina* species contains: *R. arabum* – norstictic and salazinic acids; *R. geniculata* – salazinic and sekikaic acids; *R. glaucescens* – homosekikaic, lecanoric, and sekikaic acids; *R. inflaca* and *R. unilateralis* – divaricatic acid; *R. pacifica* – protocetraric acid; and *R .peruviana* – homosekikaic, ramalinolic, and sekikaic acids[50]. Diffractaic, orsellinic, and usnic acids were isolated from *R. subcomplanata* (Nepal)[51].

All samples of *R. lacera* produced the same aromatic acids but in different amounts. Some biological activities of isolated aromatic compounds from *R .lacera* have also been reported. Thus, we recently reported that orsellic, lecanoric, diffractaic, protocetraric, usnic and norstictic acids from *R. lacera* possess antibacterial and antifungal activities[45]. Diffractaic acid exhibited antifungal activity against the phytopathogenic fungus *Cladosporium sphaerospermum*[52]. Potent antiproliferative agents, usnic and diffractaic acids showed inhibitory activities against the human keratinocyte cell line HaCaT, with IC_{50} values of 2.1 and 2.6 μM, respectively[53]. Diffractaic acid also showed strong inhibitory activity against tumor promoter-induced Epstein-Barr virus[54]. Orsellic acid revealed antibacterial activity against *Escherichia coli, Ralstonia solanacearum, Staphylococcus aureus*, and *Xanthomonas campestris vesicatoria*[55]. Protocetraric acid showed activity against yeasts *Candida albicans* and *C. glabrata*[56], and norstictic acid was active against *Aeromonas hydrophila, Bacillus subtilis, Listeria monocytogenes, Proteus vulgaris, Staphylococcus aureus, Streptococcus faecalis, Candida albicans*, and *C. glabrata*[56]. (+)-Usnic acid and (-)-usnic acid isolated from the lichen *Ramalina farinacea* showed cytotoxic and genotoxic activities against V-79 (Chinese hamster lung fibroblast-like) and A549 (human lung carcinoma epithelial-like) cell lines[57]. Usnic acid exhibited antiviral, antiprotozoal, antiproliferative, anti-inflammatory and analgesic activities as reported in recent review article[58].

Thus, the analyses of fatty acids, lipids and aromatic compounds from three samples of the *R. lacera* showed the presence of rare fatty acids: 16:4(n-3) and 18:4(n-3). Such acids were found in some marine algal species[59]. As the photobiont component may form the main part of lichens, the presence of the above acids is to be expected for lichens. Hexadeca-4,7,10,13-tetraenoic acid 16:4(n-3), octadeca-6,9,12,15-tetraenoic acid (stearidonic acid), 18:4(n-3), and α-linolenic acid were isolated from marine green alga *Ulva fasciata* (family Ulvaceae)[60]. These polyunsaturated fatty acids (PUFAs) showed potent algicidal activity against microscopic high toxic alga *Heterosigma akashiwo* (LC_{50} 1.35 μg/mL, 0.83 μg/mL, and 1.13 μg/mL for (16:4, 18:4, and α-linolenic acid, respectively), and the result demonstrated the potential of these PUFAs for practical harmful algal bloom control. These polyunsaturated acids isolated from the diatom *Navicula delognei* f. *elliptica*, showed significant antibacterial activity against *Staphylococcus aureus*, *S.epidermidis*, *Salmonella typhimurium*, and *Proteus vulgaris*[61]. α-Linolenic acid [18:3(n-3)] and oleic acid [18:1(n-9)] were found as major unsaturated fatty acids. α-Linolenic acid showed anti-inflammatory activity[62]. Among polar lipids, both betaine ether lipids (DGTA and DGTS) as well as PC, and PI were found as major lipid components.

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