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# Chemical Constituents of the Epiphytic and Lithophilic Lichens of the Genus *Collema*

**Marina Temina,[1] Dmitri O. Levitsky[2] and Valery M. Dembitsky[3*]**

[1]*Biodiversity and Biotechnology Center of Cryptogamic Plants and Fungi, Institute of Evolution, Haifa University, Haifa 31905, Israel*

[2] *CNRS UMR 6204, Biotechnologie, Biocatalyse et Biorégulation, Faculté des Sciences et des Techniques, Université de Nantes, 44037 Nantes Cedex 03, France*

[3] *Institute for Drug Research, P.O. Box 12065, Hebrew University, Jerusalem, 91120, Israel*

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**Abstract:** Biodiversity of secondary lichen metabolites by the epiphytic and lithophilic lichens of the genus *Collema* is reported. The most abundant fatty acids were α-linolenic acid (18:3n-3), oleic acid (18:1n-9), and palmitic acid (16:0), but a great variation of the ester composition from one to another was found. A comparison of neutral lipids, glycolipids, polar lipids and fatty acid composition of four species was done. DGTS, DGTA, PC, and PI were found as major components among polar lipids. Chemical constituents were characterized by GC-MS, HPLC, HR-TLC, and other chemical methods. Biological activity of isolated compounds from *Collema* species is also discusses. Several indole alkaloids and other nitrogen-containing metabolites have been isolated from the genus *Collema*: C. *cristatum, C. callopismum, C. fuscovirens* and *C. flaccidum.*

**Keywords**: *Collema*; fatty acids; polar lipids; nitrogen-containing metabolites, indole alkaloids

## 1. Introduction

Lichens, a symbiotic relationship between fungi and photosynthetic algae (and/or cyanobacteria), produce a variety of secondary metabolites [1]. Lichens have a variety of different growth forms (crustuse, foliose, fruticose). As adaptations for life in marginal habitats, lichens produce a lot of (more than 1,500) unique chemical compounds [2]. These organisms produce *n*-alkane, unusual betaine and glycolipids, unsaturated, oxygenated, branched, and halogenated fatty acids [3-11]. Lichen substances have many ecological roles, including antibiotic, antibacterial, antiviral, anti-inflammatory, analgesic, antipyretic, anti-proliferative and other activities [1,5,12-14].

* Corresponding author: E-Mail: dvalery@cc.huji.ac.il; Phone/Fax: +972-2-590-2947.

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Lichens, along with bryophytes and liverworts – create a unique epiphytic/epixylic ecological community upon trees [15,16], and/or lithophilic lichens living on rocks [17]. Epiphytic lichens in urban and rural areas can be used as indicators of the levels of pollution associated with increasing population in megapolises [18]. Also, lichen communities can be used as biomonitors of change in forest ecosystems, relating to changes in air quality, climate, and forest management over large regions and long time periods [19].

In this study, we revealed which secondary metabolites are produced by the four epiphytic and lithophilic lichen species belonging to the genus *Collema*, which growing on trees and rocks, respectively.

## 2. Materials and Methods

### *2.1. Plant Material*

The lichenized ascomycete *Collema cristatum* (L) F.H. Wigg (voucher specimen HAI-031511) was collected in January from sun exposed rock surfaces around Jerusalem at about 700 m above sea level. *Collema callopismum* A. Massal (voucher specimen HAI-0305184MT), is a foliose lichen growing on calcareous rocks. *Collema flaccidum* (Ach.) Ach (voucher specimen HAI-030514MT), and *Collema fuscovirens* (With.) J.R. Laundon (voucher specimen HAI-030512MT) belonging to fruticose and foliose growth forms, were collected on trees. These three other lichens were collected in the forests on Mount Carmel at 800 meters above sea level. All samples were collected in July from Mount Carmel, Sekher Pool (North Israel), identified and deposited in the lichen herbarium of Biodiversity and Biotechnology Center of Cryptogamic Plants and Fungi (Haifa). Clearly fresh lichen (50-110 g of each sample) was extracted (Soxhlet) with ethanol-water-HCl (90:10:1, v/v/v; 60°C) during 72 h. The ethanolic residue was further extracted by light petroleum, and then dichloromethane. All fractions were evaporated to dryness at 5°C under reduced pressure, and then dissolved in 2 ml of a cold mixture ethanol-dichloromethane (1:1, v/v) [20,21]. This solution was used for separation by HPLC, TLC, and followed chemical analysis.

### *2.2. Gas Chromatography-Mass Spectrometry*

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 as described [22,23]: the RTX 1 column (30 m, ID 0.32 mm, film thickness 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 settings was as follows: initial temperature, 40°C; initial time, 2 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 7$^{th}$, and NIST-98). Standard nitrogen-containing compounds were obtained from Sigma-IL.

### *2.3. 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 [24,25]. Neutral lipids were separated on 10 x 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 [10,26]. 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 [27,28].

### *2.4. 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 detector 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 x 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 [29].

## 3. Results and Discussion

Four epiphytic and lithophilic species belonging to the genus *Collema* (family Collemataceae) were chosen for a comparative examination and to identify some chemicals in these groups of their fatty acid, polar lipids, and aromatic compounds. The GC-MS analysis of fatty acids in *Collema*, which grows on calcareous rocks and trees, revealed a high polyenoic content in species which grows on the Palestine oak (*Quercus calliprinos*) (62.1 and 61.2%; Table l), but much lower amounts of such acids in two other *Collema* species, viz. 22.5 and 19.2%, respectively. The amounts of trienoic acids, however, were higher in the samples from tree-growing species, 43.9% (No 3), and 42.7% (No 4). Strong discrepancy of saturated fatty acids between species growing on rocks and trees were identified with *n*-16:0 as major one (56-58% and 12-13%, respectively). All species studied had almost identical monoenoic acid contents. Dienoic and trienoic acids had also strong discrepancy, as well as the saturated acids.

Tetraenoic and pentaenoic acids have not been found in *Collema* species growing on rocks, and they are present in species growing on the trees (Table 1). Among other interesting acids 4,7,10,13-hexadecatetraenoic acid [16:4(n-3)], the presence of which is characteristic for green marine macrophytic [7,30], and microscopic algae [31], and stearidonic acid [18:4(n-3)], characteristic for brown marine algae [32,33], were detected. It seems likely that the lichen photobiont synthesize these acids [3], though two species (No 1 and No 2) do not synthesize these acids. The total level of isomers of arachidonic acid [20:4(n-6) and 20:4(n-3)] in two species was considerably higher than that from the all of the examined species, reaching over 5%. 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 the individual lipid classes, for example, *Peltigera aphthosa* [5].

Total lipid content has been studied in all collected lichen species, having different climatic peculiarities, and substrates. Table 2 shows total lipid compositions in lichens collected during January (No 1), and in July (No 2,3, and 4); total lipid content in such lichens show variations from 28.8-66.8 mg/g dry wt. Neutral lipids make up the highest percentage among of total lipids (Table 2) and vary from 15.8-36.6 mg/g dry wt.

Study of the lichen polar lipids, including betaine lipids and phospholipids showed DGTA (diacylglyceryltrimethylalanine), DTGS diacylglyceryltrimethylhomoserine), and PC (phosphatidyl-choline) to be the major polar lipids with concentrations varying from 71.5 to 83.9% of total polar lipids (Table 2). The PC content in various fungal species is known to vary from 19 to 50% [34], whereas PC contents in red algal species varies up to 78% [35]. PE was detected in all lichen species

studied; its level was low in the first two species (ca 3-5%), but reached 11-12% in species No 3 and No 4. PI was also found in all species studied; its level was highest in No 4 (26.7%).

**Table 1.** Main fatty acids of four lichen species of the genus *Collema*

| Fatty acids | **1** | **2** | **3** | **4** |
|---|---|---|---|---|
| ***Saturated*** | **56.8** | **58.9** | **13.6** | **13.0** |
| 12:0 | 3.2 | 2.7 | 0.5 | |
| 13:0 | 1.0 | 1.1 | 0.6 | 0.7 |
| 14:0 | 5.2 | 4.2 | 0.9 | 0.9 |
| 15:0 | 1.2 | 1.0 | 0.7 | 0.5 |
| *i*-15:0 | 1.8 | 1.3 | 0.7 | 0.5 |
| *ai*-15:0 | 1.6 | 3.0 | 0.5 | 0.6 |
| 16:0 | 34.1 | 38.3 | 6.9 | 6.0 |
| *i*-17:0 | 1.7 | 1.8 | 0.5 | 0.6 |
| *ai*-17:0 | 1.8 | 1.7 | 0.5 | 0.7 |
| 18:0 | 4.0 | 2.5 | 1.3 | 1.9 |
| 20:0 | 1.2 | 1.3 | 0.5 | 0.6 |
| ***Monoenes*** | **14.8** | **14.9** | **13.9** | **11.6** |
| 15:1(*n*-8) | | | 0.7 | 0.5 |
| 16:1(*n*-9) | 0.7 | 0.5 | 2.9 | 1.7 |
| 16:1(*n*-7) | 0.8 | 0.6 | 1.9 | 1.9 |
| 18:1(*n*-11) | 0.9 | 0.8 | 0.6 | 0.6 |
| 18:1(*n*-9) | 11.0 | 11.4 | 6.4 | 5.8 |
| 18:1(*n*-7) | 1.4 | 1.0 | 0.8 | 0.6 |
| 20:1(*n*-9) | | 0.6 | 0.6 | 0.5 |
| ***Dienes*** | **5.9** | **5.6** | **10.4** | **14.2** |
| 16:2(*n*-4) | 0.5 | 0.7 | 0.7 | 2.1 |
| 18:2(*n*-6) | 4.8 | 4.2 | 9.1 | 10.3 |
| 20:2(*n*-6) | 0.6 | 0.7 | 0.6 | 1.8 |
| ***Polyenes*** | **22.5** | **19.2** | **62.1** | **61.2** |
| 16:3(*n*-6) | 1.1 | 0.9 | 3.1 | 4.1 |
| 16:3(*n*-3) | 1.6 | 0.7 | 3.7 | 3.9 |
| 18:3(*n*-6) | 2.2 | 1.9 | 4.1 | 4.0 |
| 18:3(*n*-3) | 17.5 | 16.6 | 33.3 | 30.7 |
| 16:4(*n*-3) | ND | ND | 4.3 | 5.1 |
| 18:4(*n*-3) | ND | ND | 4.2 | 4.7 |
| 20:4(*n*-6) | ND | ND | 1.9 | 2.1 |
| 20:4(*n*-3) | ND | ND | 3.2 | 2.9 |
| 20:5(*n*-3) | ND | ND | 4.3 | 3.7 |

ND, not detected. Lithophilic species: **1:** *Collema cristatum*, and **2:** *Collema callopismum*, both were determined on calcareous rocks. Epiphytic species: **3**: *Collema flaccidum*, and **4:** *Collema fuscovirens*, both were determined on trees.

Both betaine lipids were detected in all samples. Their level was high in species No 1 and 2 (45-47%). DGTS, one of the three known betaine lipids, has been the object of many studies [36]. Betaine lipids occur in bacteria [37], fungi [38], moss species [39], and in a number of brown, green and red algae [7,24,26,40] as well as in lichens [41,42]. As for higher plants, betaine lipids have been found in bryophytes [16,38], in ferns [27], and other plants [25]. DGTA was also detected in fungi [36,38], and marine brown algae [24,36].

Thus, the analyses of fatty acids, lipids and aromatic compounds from two species of the genus *Collema* 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 [30]. As the photobiont component may form the main part of lichens, the presence of the above acids is to be expected for lichens [3,43]. 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) [44]. 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* [45]. α-Linolenic (18:3n-3) and oleic (18:1n-9) acids were found as major unsaturated fatty acids. α-Linolenic acid (α-LA) showed anti-inflammatory activity [46]; α-LA for 4 wk have cardioprotective effects similar to the effects of fish oil [47]. Among polar lipids, both betaine lipids (DGTA and DGTS) as well as PC, and PI were found as major lipid components.

**Table 2.** Lipid composition of four lichen species of the genus *Collema*

| Lipid classes | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Total lipids (mg/g dry wt) | 28.8 | 29.4 | 66.8 | 64.4 |
| Neutral lipids (mg/g dry wt) | 17.5 | 15.8 | 36.6 | 30.2 |
| Free fatty acids[#] | TR | 0.9±0.2 | 2.9±0.4 | 2.9±0.3 |
| Free sterols | 2.6±0.4 | 1.6±0.4 | 4.6±0.2 | 2.6±0.2 |
| Diacylglycerols | 1.1±0.1 | 1.9±0.7 | 3.1±0.3 | 2.1±0.3 |
| Triacylglycerols | 8.3±0.6 | 8.0±0.8 | 12.3±0.9 | 14.0±1.1 |
| Steryl esters | 5.5±0.2 | 2.2±0.5 | 6.2±0.5 | 5.4±0.7 |
| Wax esters | TR | 1.2±0.3 | 3.0±0.6 | 3.2±0.5 |
| | | | | |
| Glycolipids (mg/g dry wt) | 6.9 | 8.0 | 21.1 | 26.6 |
| MGDG | 2.0±0.3 | 2.2±0.3 | 3.2±0.3 | 10.4±0.5 |
| DGDG | 4.3±0.6 | 5.3±0.4 | 7.2±0.5 | 12.9±0.8 |
| SQDG | 0.6±0.1 | 0.5±0.1 | 0.9±0.1 | 3.3±0.3 |
| | | | | |
| Polar lipids (% of total polar lipids) | 4.4 | 5.6 | 9.1 | 7.6 |
| DGTA | 27.1±0.9 | 28.3±1.9 | 2.8±0.5 | 1.9±0.4 |
| DGTS | 19.9±1.1 | 16.8±1.2 | 8.1±0.9 | 3.3±0.6 |
| PC | 27.8±1.8 | 29.4±2.5 | 62.1±2.8 | 66.3±4.7 |
| PE | 3.8±0.4 | 5.2±0.6 | 10.3±0.9 | 11.8±0.9 |
| PI | 14.8±0.8 | 11.2±0.8 | 16.7±0.7 | 26.7±1.7 |
| PA | ND | 1.9±0.1 | ND | ND |
| X | 6.6±0.9 | 7.2±0.6 | ND | ND |

[#]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; ND, not detected, TR, trace

A known photo protective mycosporine (Figure 1) named Collemin A [29], was also isolated from the *C.cristatum* and *C. callopumum* by HPLC, both lichens growing on calcareous rocks. It was found that the pure isolated compound prevents UV-B induced cell destruction in a dose-dependent manner, partially prevents pyrimidine dimer formation and completely prevents UV-B induced erythema when applied to the skin prior to irradiation. Species of *C. flaccidum*, and *C. fuscovirens* growing on trees did not produce this metabolite. Other nitrogen-containing compounds were also detected in lichens (Table 3) using HPLC and/or GC/MS.

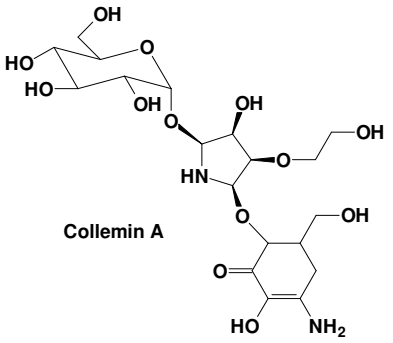


**Figure 1**. Biological active photo protective alkaloid, Collemin A, isolated from the *Collema cristatum*

Indole-3-acetic acid was detected in many lichen species: *Canoparmelia caroliniana, C. crozalsiana, C. texana, Parmotrema sancti-angeli* and *P. tinctorum* [48]; *Dermatocarpon intestiniforme, Flavoparmelia caperata, Lecanora muralis, Neofuscelia pulla, Rhizocarpon geographicum, Tephromela atra*, and *Xanthoria elegans* [49]; and also in the genera *Cladonia, Parmelia, Peltigera*, and *Usnea* [50]. Tryptophan was isolated from *Schizophyllum commune* [51]. Other nitrogen-containing compounds were found for the first time in lichen species.

**Table 3.** Nitrogen-containing compounds detected in the genus *Collema**

| Lichen Metabolites | **1** | **2** | **3** | **4** |
|---|---|---|---|---|
| Collemin A | 114.3 | 29.8 | ND | ND |
| Indole-3-acetic acid | 49.9 | 22.1 | 28.9 | 33.7 |
| Bufotenine | ND | ND | 14.2 | 2.9 |
| 5-Hydroxy-N-methyltryptamine | ND | ND | 4.8 | 18.3 |
| 5-Hydroxytryptophan | ND | ND | 11.3 | 6.9 |
| Serotonin | ND | 4.9 | 6.9 | ND |
| Tryptamine | 5.2 | 3.2 | ND | ND |
| Tryptophan | 2.6 | 3.9 | 11.7 | 17.9 |

*μg/100 g dry wt; ND: not detected

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