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MOLECULAR STRUCTURAL STUDIES OF LICHEN SUBSTANCES WITH ANTIMICROBIAL, ANTIPROLIFERATIVE, AND CYTOTOXIC EFFECTS FROM *Parmelia subrudecta*

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□ *Lecanoric acid (***1***), orsellinic acid methyl ester (***2***), orcinol (***3***), and usnic acid (***4***) were isolated from the lichen* Parmelia subrudecta, *collected on Palma of the Canary Islands, Spain. Compounds* **1, 2, 3,** *and* **4** *were purified by solvent extraction, silica gel column chromatography, and preparative high-performance liquid chromatography (HPLC) consecutively. The structures of the four compounds were elucidated by one- and two-dimensional nuclear magnetic resonance (NMR) experiments and mass spectrometric investigations. These compounds showed activity against important gram-positive and gram-negative pathogens like mycobacteria and multiresistant staphylococci. This activity is combined with antiproliferative activity and cytotoxicity.*

Keywords antiproliferative and cytotoxic effects, bioresources, lecanoric acid, lichens, orcinol, orsellinic acid methyl ester, *Parmelia subrudecta*, usnic acid

INTRODUCTION

The lichens are a unique life form that depends upon the symbiotic relationship between algae and fungi. Together this association produces some of the most hardy organisms on earth. The chemistry of lichen constituents has been studied extensively for more than a century. Edwards et al. (2003) reported that the Raman spectra of the major products of lichen metabolism show evidence for three groups of products, namely, polysaccharides and pulvinic acid (from the shikimic acid pathway), terpenes and carotenoids (from the mevalonic acid pathway) and anthraquinones, and

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usnic acid, orsellinic acid, and depsides (from the acetyl-polymalonyl pathway).[1]

Based on the presence of orbicular pseudocyphellae and simple rhizines, some members of genus *Parmelia* s. str. are taxonomically treated as members of genus *Punctelia*,[2] including the species *Parmelia borreri* and *Parmelia subrudecta*. Both species have similar habitus; however, *Parmelia borreri* has a lower surface black in the center, while *Parmelia subrudecta* is characterized by pale brown lower surface at the center.[3] Differences between the two species are more pronounced in chemical traits, as in the medulla of *P. borreri* gyrophoric acid has been found as the dominant secondary compound, while in medulla of *P. subrudecta* lecanoric acid is the main extracellular metabolite.

In this paper, we report the isolation and structural elucidation of compounds **1, 2, 3,** and **4**, based on spectral analyses and chemical correlations, and their biological activities (antimicrobial, antiproliferative, and cytotoxic).

EXPERIMENTAL

Medicinal Lichen

The lichen *Parmelia subrudecta* Nyl. 1888 was collected on Palma of the Canary Islands, Spain. Sampling was done in 2002. Lichen was taxonomically determined by Dr. Martin Bačkor. Voucher specimens are stored in a lichen herbarium at the Department of Botany, Faculty of Science, Šafárik University, in Košice (Slovakia).

General Experimental Procedures

Electrospray mass spectrometry (ESI-MS) and high-resolution electron impact mass spectrometry (EI-HRMS) were carried out by use of a triple-quadrupole mass spectrometer (VG Biotech Altrincham, England) and a Finnigan MAT 95XL mass spectrometer (Finnigan, Bremen, Germany), respectively. NMR measurements were done on a Bruker Avance DRX 500 instrument (in CD_3OD and $CDCl_3$), and Fourier transform infrared (FTIR) spectroscopy on a Mattson Satellite instrument equipped with an ATR measuring device. The ^{13}C multiplicity data were obtained from DEPT (distortionless enhancement by polarization transfer) experiments. The chemical shifts are expressed in (values (ppm). ^{1}H-^{1}H COSY, HSQC, and HMBC were obtained by conventional methods. Ultraviolet–visible (UV-vis) spectroscopy was recorded on a Specord 2000 instrument (Analytik Jena, Germany). Infrared (IR) spectra were recorded on a Beckman DU 601 and Shimadzu IR scanning spectrophotometers. Specific rotations were determined on a Perkin-Elmer 141 polarimeter.

Extraction and Isolation of the Compounds Lecanoric Acid (1), Orsellinic Acid Methyl Ester (2), Orcinol (3), and Usnic Acid (4) From *Parmelia subrudecta*

The isolation procedure started with extraction of the dried material (20 g) of *Parmelia subrudecta* with $CHCl_3$/MeOH (1:5, v/v) or *n*-butanol for 48 hr and this was filtered. The organic layer was evaporated to dryness under reduced pressure, yielding 500 mg of crude product, which was dissolved in chloroform–methanol (1:3) and chromatographed on a silica gel 60 column (70–230 mesh). The compounds **1, 2,** and **3** were eluted from the column by $CHCl_3$/MeOH (1:3, v/v), but the compound **4** was eluted by chloroform. The active eluates of compounds **1, 2,** and **3** were concentrated in vacuo, then applied to a Sephadex (LH-20) column chromatograph and developed with methanol. The active fractions thus obtained were concentrated under reduced pressure. The final separation and purification of **1, 2,** and **3** by preparative high-performance liquid chromatography (HPLC) on silica gel RP 18 (column 25 × 1.5 cm, 5 μm, using a gradient of 95% water to 5% acetonitrile and monitoring at 210 nm (flow rate, 3.0 mL min^{-1}), delivered the pure compounds of lecanoric acid **1** (15 mg), orsellinic acid methyl ester **2** (10 mg), and orcinol **3** (7.2 mg) as colorless solids. The active chloroform eluate of compound **4** was concentrated in vacuo. The identification of usnic acid was further confirmed by thin-layer chromatography (TLC) developed in the solvent system of chloroform. The spot was also visualized by 0.5% solution of vanillin in methanol/sulfuric acid/acetic acid.

Analytical HPLC

Analytical HPLC/diode array analysis of compounds **1, 2,** and **3** was carried out on Nucleosil 100, C 18 (column 125 × 4.6 mm, 5 μm, using a gradient of acetonitrile in water/0.1% trifluoroacetic acid (TFA) and monitoring at 210 and 230 nm (diode array detector) with a flow rate of 1.0 mL min^{-1}. Compounds **3, 2,** and **1** were eluted with retention times (t_R) of 13.4, 15.0, and 17.5 min.

Thin-Layer Chromatography (TLC)

TLC was carried out on silica gel plates (Merck 60, F_{254}) with chloroform–methanol (9.0:1.0, v/v). The chromatographic spots were visualized by spraying with a 0.5% solution of vanillin in methanol/sulfuric acid/acetic acid and heating at 120°C for 3–5 min. In this system the compounds **1, 2,** and **3** possessed an R_f value of 0.15, 0.66, and 0.37, respectively, and gave a red color. Compound **4** in this system possessed an R_f value of 0.78 (blue color).

Antimicrobial Activity

Antimicrobial activity was determined by the agar diffusion test according to the European Pharmacopoia of 1997.[4]

Antiproliferative and Cytotoxic Assay

Antiproliferative and cytotoxic assay was determined according to reference.[5]

RESULTS AND DISCUSSION

Compound **1** was obtained as a colorless plate crystals, which was soluble in lower alcohols, dimethyl sulfoxide, *N,N*-dimethylformamide. and ethyl acetate, but insoluble in water, ether, and *n*-hexane. It gave a red color reaction with a 0.5% solution of vanillin in methanol/sulfuric acid/acetic acid and $FeCl_3$ reagents, but not with ninhydrin and Sakaguchi reagents. The UV-vis spectra showed absorption maxima at 212, 269, and 301 nm by HPLC analysis (diode array detector). The IR spectrum with KBr showed absorption maxima at 3415, 2970, 1654, 1587, 1484, 1460, 1435, 1378, 1313, 1284, 1251, 1204, 1142, 1070, 900, 826, 691, and 592 cm^{-1}. Compound **1** decomposed at 175°C and had no optical activity.

The structures of compounds **1, 2,** and **3** were settled on the basis of electrospray (ESI-MS), high-resolution electron impact mass spectrometry (EI-HRMS), and extensive NMR studies (^1H, ^{13}C, DEPT 135, ^1H-^1H COSY, HSQC, and HMBC).

The (+)-ESI mass spectrum of **1** displayed pseudo-molecular ions at m/z 341.1 $(M+Na)^+$ and in the negative ion mode at m/z 317.2 $(M-H)^-$. The molecular mass (318 Da) and the chemical formula $C_{16}H_{14}O_7$ were readily determined by high-resolution electron impact mass spectrometry due to m/z 318.2780. This formula indicated 10 double-bond equivalents.

In the ^1H-NMR spectrum of compound **1**, the low-field signals at δ_H 6.21, 6.27, 6.43, and 6.48 were peculiar to four aromatic protons. Furthermore, the protons of two methyl groups were observed at δ_H 2.56 and 2.62.

In the ^{13}C NMR and DEPT spectra of **1,** all 16 carbon signals were visible (Table 1) and were assignable to 2 carbonyl carbons (δ_C 171.2 and 175.8), 12 aromatic carbons (4 CH and 8 C_q signals), and 2 aromatic methyl carbons (δ_C 23.5 and 24.3). The chemical shifts of all proton and carbon atoms of **1** are summarized in Table 1.

A ^1H-^1H chemical shift correlation spectroscopy (COSY) experiment demonstrated coupling only from the aromatic protons H-5 and H-5́at δ_H 6.43 and 6.27 to the methyl protons H_3-8 (δ_H 2.62) and to H_3-8́(δ_H 2.56).

TABLE 1 ^{1}H and ^{13}C NMR Chemical Shifts of Lecanoric Acid **(1)** in CD_3OD (600 MHz and 125 MHz, TMS as Internal Standard, Chemical Shifts in δ Values)

Assignment	δ_H, J (Hz)	HMBC (H→C)	δ_C	Groups
1	—	—	118.1 s	Cq
2	—	—	164.6 s	Cq
3	6.48 (1H, d, 3.0 Hz)	C-1, C-2, C-4, C-5	108.3 d	HC=
4	—		152.9 s	Cq
5	6.43 (1H, d, 2.0 Hz)	C-1, C-3, C-4, C-8	115.7 d	HC=
6	—		144.5 s	Cq
7	—		171.2 s	C=O
8	2.62 (3H, s)	C-1,C-5,C-6,C-7	23.5 q	CH_3
1′	—		105.3 s	Cq
2′	—		164.9 s	Cq
3′	6.21 (1H, d, 2.0 Hz)	C-1′,C-2′,C-4′,C-5′	102.0 d	HC=
4′	—		166.6 s	Cq
5′	6.27 (1H, d, 3.0 Hz)	C-1′, C-3′, C-8′	113.1 d	HC=
6′	—		144.7 s	Cq
7′	—		175.8 s	C=O
8′	2.56 (3H, s)	C-1′, C-5′, C-6′, C-7′	24.3 q	CH_3

Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet.

The heteronuclear multiple-bond correlation (HMBC) experiment of compound **1** is demonstrated on Table 1.

On the basis of the obtained data (Table 1) it was established that compound **1** was identical with lecanoric acid (Figure 1).[6–8] Lecanoric acid is a common medullary lichen depside. Umezawa et al. (1974) demonstrated that lecanoric acid is an inhibitor of histidine decarboxylase with extremely low toxicity.[9] Umezawa et al. (1984) showed inhibition of tumor promotion by a lecanoric acid analogue.[10]

Compound **2** was obtained as colorless microcrystals, which were soluble in lower alcohols, dimethylsulfoxide, *N,N*-dimethylformamide, and ethyl acetate, but insoluble in water, ether, and *n*-hexane. It gave a red color reaction with a 0.5% solution of vanillin in methanol/sulfuric acid/acetic acid and $FeCl_3$ reagents. Compound **1** decomposed at 140°C and had no optical activity.

The (+)-ESI mass spectrum of **2** displayed pseudo-molecular ions at m/z 409.3 $(2M + 2Na)^+$ and in the negative ion mode at m/z 181.0 $(M - H)^-$. The molecular mass (182 Da) and the chemical formula $C_9H_{10}O_4$ were readily determined by high-resolution electron impact mass spectrometry due to m/z 182.05784. This formula indicated five double-bond equivalents.

In the ^{1}H-NMR spectrum of compound **2**, the low-field signals at (δ_H 6.13 and 6.18 were peculiar to two aromatic protons. Furthermore, the proton signals of one methyl group and one methoxy group were observed at δ_H 2.43 and 3.89.

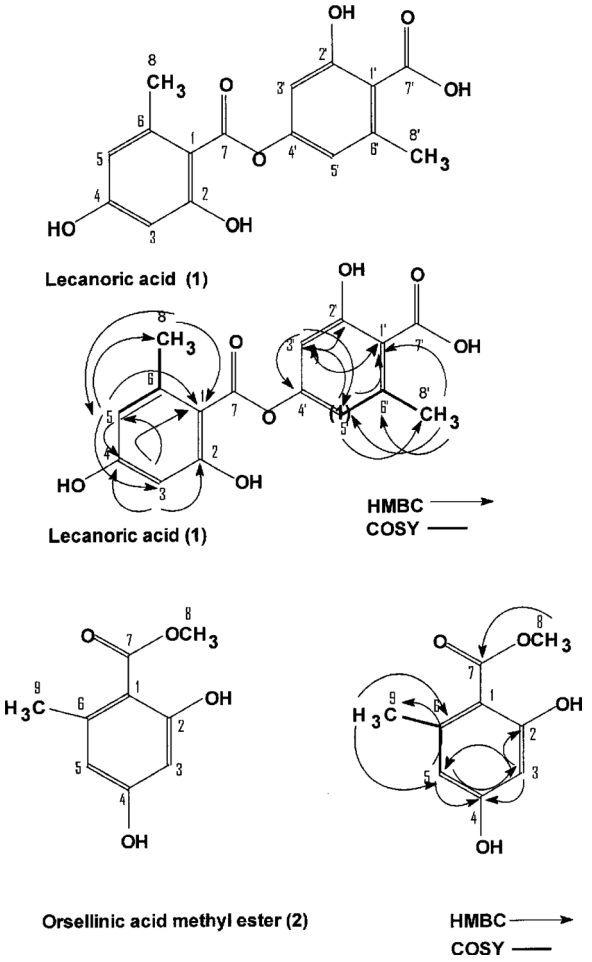

FIGURE 1 Structures of lichen compounds **1** and **2** from *Parmelia subrudecta*.

In the ^{13}C-NMR and DEPT spectra of **2** all nine carbon signals were visible (Table 2), and were assignable to one carbonyl carbon (δ_C 173.8), six aromatic carbons (2 CH and 4 C_q signals), one aromatic methyl (δ_C 24.2), and one methoxy (δ_C 52.1) carbon signal. The chemical shifts of all proton and carbon atoms of **2** are summarized in Table 2. For the structural assignment of compound **2**, the ^{1}H-^{1}H COSY and ^{1}H-^{13}C long-range heteronuclear coupled NMR spectra (HMBC) were of pivotal importance. The ^{1}H-^{1}H COSY experiment demonstrated also coupling from the aromatic proton H-5 at δ_H 6.18 to the methyl protons H3-9 (δ_H 2.43).

A heteronuclear multiple bond correlation (HMBC) experiment showed long-range couplings from proton H-3 at δ_H 6.13 to the quaternary carbons C-2 at δ_C 164.6 and C-4 at δ_C 166.2 and the aromatic carbon C-5 at

TABLE 2 ^{1}H and ^{13}C NMR Chemical Shifts of Orsellinic Acid Methyl Ester (**2**) in CD_3OD (600 MHz and 125 MHz, TMS as Internal Standard, Chemical Shifts in δ Values)

Assignment	δ_H, J (Hz)	HMBC (H→C)	δ_C	Groups
1	—		116.0 s	Cq
2	—		164.6 s	Cq
3	6.13 (1H, d, 3.0 Hz)	C-2, C-4, C-5	101.9 d	HC=
4	—		166.2 s	Cq
5	6.18 (1H, d, 1.0 Hz)	C-3, C-4, C-9	112.9 d	HC=
6	—		144.4 s	Cq
7	—		173.8 s	C=O
8	3.89 (3H, s)	C-7	52.1 q	OCH_3
9	2.43 (3H, s)	C-6, C-5	24.2 q	CH_3

Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet.

δ_C 112.9. Similar couplings were also observed from H-5 (δ_H 6.18) to the quaternary carbon C-4 (δ_C 166.2), the aromatic carbon C-3 (δ_C 101.9), and methyl carbon C-9 at δ_C 24.2. Cross peaks were also observed from the methyl protons at δ_H 2.43 to the aromatic proton C-5 at δ_C 112.9 and the quaternary carbon C-6 at δ_C 144.4. The remaining singlet signal of H-8 (δ_H 3.89) showed an HMBC correlation to the carbonyl carbon C-7 (δ_C 173.8). Thus, the planar structure of compound **2** was unequivocally determined as orsellinic acid methyl ester (Figure 1).[11]

The (+)-ESI mass spectrum of the isolated from *Parmelia subrudecta* compound **3** displayed pseudo-molecular ions at m/z 271.6 $(2\,M + Na)^+$ and 497.1 $(4\,M + H)^+$. The molecular mass (124 Da) and the chemical formula $C_7H_8O_2$ were readily determined by high-resolution electron impact mass spectrometry due to m/z 124.05229. This formula indicated four double-bond equivalents. Compound **3** was soluble in lower alcohols, dimethyl sulfoxide, and *N,N*-dimethylformamide, but insoluble in water, ether, and *n*-hexane. Compound **3** decomposed at 107.5°C.

In the ^{1}H-NMR spectrum of compound **3**, the low-field signals at (δ_H 6.05 and 6.11 were peculiar to three aromatic protons. Furthermore, the protons of one methyl group were observed at δ_H 2.26.

In the ^{13}C-NMR and DEPT 135 spectra, all seven carbon signals were visible (Table 3), and were assignable to six aromatic carbons (3 CH and 3 C_q signals) and one aromatic methyl (δ_C 21.6) carbon signal. The chemical shifts of all proton and carbon atoms of **3** are summarized in Table 3. The ^{1}H-^{1}H COSY experiment demonstrated also coupling from the aromatic proton H-5 at δ_H 6.11 to the methyl protons H_3-7 (δ_H 2.26).

The HMBC experiment (Table 3) showed additionally a long-range coupling from the aromatic doublets of H-1 and H-5 at δ_H 6.11 to quaternary carbons C-2 and C-4 at δ_C 159.3. Similarly, a coupling was established from the aromatic proton of H-3 at δ_H 6.05 to the quaternary carbons

TABLE 3 ^{1}H and ^{13}C NMR Chemical Shifts of Orcinol (**3**) in CD_3OD (600 MHz and 125 MHz, TMS as Internal Standard, Chemical Shifts in δ Values)

Assignment	δ_H, J (Hz)	HMBC (H→C)	δ_C	Groups
1	6.11 (1H, d, 2.0 Hz)	C-2, C-3	108.6 d	HC=
2	—		159.3 s	Cq
3	6.05 (1H, d, 2.0 Hz)	C-1, C-2, C-4, C-5	100.8 d	HC=
4	—		159.3 s	Cq
5	6.11 (1H, d, 2.0 Hz)	C-3, C-4	108.6 d	HC=
6	—		141.2 s	Cq
7	2.26 (3H, s)	C-1, C-5, C-6	21.6 q	CH_3

Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet.

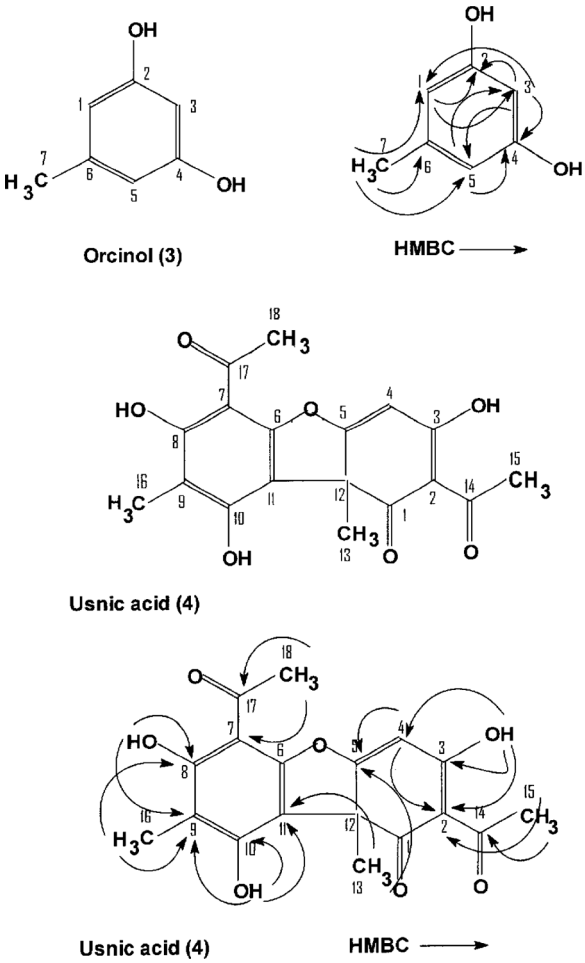

FIGURE 2 Structures of lichen compounds **3** and **4** from *Parmelia subrudecta*.

C-2 and C-4 at δ_C 159.3 and the aromatic carbons of C-1 and C-5 (δ_C 108.6). Cross peaks were also observed from the methyl protons at δ_H 2.26 to the aromatic carbons of C-1 and C-5 at δ_C 108.6 and the quaternary carbon C-6 at δ_C 141.2. On the basis of these data, the structure of compound **3** (Figure 2) was finally elucidated as orcinol. Compound **3** (orcinol) for the first time was isolated from the culture filtrate of *Aspergillus fumigatus* and *Gliocladium roseum*. Orcinol is a reagent for the determination of aromatic aldehydes and of carbohydrates.[12]

Compound **4** was isolated as yellow microcrystals. It is soluble in ethyl acetate and chloroform, but insoluble in lower alcohols and water. The UV-vis spectrum showed absorption maxima at 232 and 283 nm. The molecular mass (344 Da) and the chemical formula $C_{18}H_{16}O_7$ were readily determined by EI-HRMS due to m/z 344.08850. The formula suggested the presence of 11 double bonds or rings in the molecule. The identification of compound **4**, isolated from the lichen *Parmelia subrudecta*, was further confirmed by TLC analysis and by one- and two-dimensional NMR experiments (Table 4). The presence of usnic acid was determined by silica gel thin-layer chromatography developed with chloroform. The spot was detected by spraying with a 0.5% solution of vanillin in methanol/sulfuric acid/acetic acid and heating at 120°C for 3–5 min (blue

TABLE 4 ^1H and ^{13}C NMR Chemical Shifts of Usnic Acid **(4)** in $CDCl_3$ (600 MHz and 125 MHz, TMS as Internal Standard, Chemical Shifts in δ Values)

Assignment	δ_H, J (Hz)	HMBC (H→C)	δ_C	Groups
1	—		198.2 s	C=O
2	—		105.3 s	Cq
3			191.7 s	Cq
3-OH	18.85 (1H, s)	C-2, C-3, C-4		
4	5.98 (1H, s)	C-2, C-5	98.3 d	HC=
5	—		179.4 s	Cq
6	—		155.2 s	Cq
7	—		101.5 s	Cq
8	—		163.9 s	Cq
8-OH	13.30 (1H, s)	C-8, C-9		
9	—		109.4 s	Cq
10	—		157.5 s	Cq
10-OH	11.00 (1H, s)	C-9, C-10, C-11		
11	—		103.9 s	Cq
12	—		59.1 s	Cq
13	1.75 (3H, s)	C-5, C-11	32.1 q	CH_3
14	—		201.7 s	C=O
15	2.67 (3H, s)	C-2, C-14	27.5 q	CH_3
16	2.15 (3H, s)	C-8, C-9, C-10	7.5 q	CH_3
17	—		200.3 s	C=O
18	2.67 (3H, s)	C-7, C-17	31.2 q	CH_3

Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet.

TABLE 5 Antimicrobial Activity of Usnic Acid (**4**) in the Agar Diffusion Assay

	Test organisms	Diameter of inhibition zones (mm)
1	*Bacillus subtilis* ATCC 6633	35
2	*Staphylococcus aureus* SG 511	25
3	*Staphylococcus aureus* 134/93 (MRSA)	28
4	*Staphylococcus aureus* 1528	40
5	*Sporobolomyces salmonicolor* 549	19
6	*Pseudomonas aeruginosa* SG 137	12
7	*Pseudomonas aeruginosa* K 799/61	18
8	*Penicillium notatum* JP 36	13
9	*Escherichia coli* SG 458	12
10	*Mycobacterium smegmatis* SG 987	0
11	*Candida albicans* Bayer-Ruck	0

color). The R_f value = 0.31 of the spot of compound **4** was compared with those of authentic standard (usnic acid, R_f value = 0.31, mobil phase chloroform, blue color). On the basis of these data, the structure of compound **4** (Figure 2) was finally elucidated as usnic acid.[13,14]

Lecanoric acid (**1**) and usnic acid (**4**) were investigated in vitro for antimicrobial activity against gram-positive and gram-negative bacteria, yeasts, and filamentous fungi. Lecanoric acid (**1**) showed activity against gram-positive bacteria such as *Staphylococcus aureus* SG 511 (MIC, 200 μg/mL), *Staphylococcus aureus* MRSA (MIC, 1000 μg/mL), and *Mycobacterium tuberculosis* (MIC, 100 μg/mL). Usnic acid was active against *Bacillus subtilis, Staphylococcus aureus, Sporobolomyces salmonicolor, Penicillium notatum, Escherichia coli,* and *Pseudomonas aeruginosa*, but was inactive against *Mycobacterium smegmatis* and *Candida albicans* Bayer-Ruck (Table 5).

The antiproliferative and cytotoxic effects of lecanoric acid (**1**), orsellinic acid methyl ester (**2**), orcinol (**3**), and usnic acid (**4**) from *Parmelia subrudecta* were determined with L-929 mouse fibroblast cells,

TABLE 6 Antiproliferative Effect and Cytotoxicity of Lecanoric Acid (**1**), Orsellinic Acid Methyl Ester (**2**), Orcinol (**3**), and Usnic Acid (**4**) Against Cell Cultures of L-929, K-562, and HeLa Cells

	Antiproliferative effect, GI_{50}[a] (μg/mL)		Cytotoxicity, CC_{50}[b] (μg/mL) (IC_5[c], μg/mL)
Compounds	L-929	K-562	HeLa
Lecanoric acid (**1**)	50.0	50.0	50.0 (50.0[c])
Orsellinic acid, methyl ester (**2**)	50.0	50.0	50.0 (2.1[c])
Orcinol (**3**)	47.7	42.3	50.0 (11.5[c])
Usnic acid (**4**)	5.5	1.4	5.1 (1.4[c])

[a]Cellular growth inhibition.
[b]Cytotoxic efficacy.
[c]Inhibition concentration 5%.

K-562 human leukemia cells, and HeLa human cervix carcinoma. The GI_{50} and CC_{50} values are summarized in Table 6.

CONCLUSION

The combination of lichen metabolites lecanoric acid (**1**), orsellinic acid methyl ester (**2**), orcinol (**3**), and usnic acid (**4**) for the first time was isolated from the lichen *Parmelia subrudecta,* collected on Palma of the Canary Islands, Spain. We did not find data about the isolation and structure elucidation of orsellinic acid methyl ester (**2),** orcinol (**3**), and usnic acid (**4**) from *Parmelia subrudecta.* The orsellinic acid methyl ester (**2**) is a new natural product, isolated from the lichen *Parmelia subrudecta.* To the best of our knowledge, orsellinic acid methyl ester (**2)** has not been isolated from any other natural source; however, it is known as a synthetic product (derivative of orsellinic acid).[11,15,16] For the first time we established that the all isolated substances have antiproliferative and cytotoxic effects against L-929, K-562, and HeLa cell lines. These compounds showed activity against important gram-positive pathogens like mycobacteria and multiresistant staphylococci.

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