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Isolation and Structure Elucidation of Novel Natural Products from Turkish Lichens

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Three natural products were isolated from three different Turkish lichens. Microbiological investigations were also examined of these lichens. Compound **1** is known as 2-hydroxy-4-methoxy-3,6-dimethyl benzoic acid, and is isolated from *Pseudevernia furfuracea* for the first time. Compounds **2** and **3** are detected as new compounds and called, 3-acetyl-4-amino-2-chloro-1-(4-hydroxy-2-methoxy- 6-methylphenyl carbonyloxy) benzene and 2-(4-methoxyphenyl)-2-(5-oxo-4-phenyl-2,5-dihydro-2-furaniliden) acetic acid; they were isolated from *Evernia prunastri* and *Letharia vulpina*, respectively. All these compounds had different skeletons. Their structures were established by chemical methods and spectroscopic techniques using IR, UV, 1D and 2D NMR and EI and (+) FAB-Mass methods.

Key Words: Turkish lichens, Pseudevernia furfuracea, Evernia prunastri, Letharia vulpina, Phenolic compounds.

Introduction

Lichens are the symbiotic association of fungi and a photosynthetic partner, either a green algae of a cynobacterium or both. The lichens' names refer to their fungal components. About 18,000 lichen species are known. They provide a great variety of metabolic products, some of which appear to occur naturally only in lichens, while others are also present in higher plants and fungi. Their secondary products play a dominant role in the systematics of lichen forming fungi. The isolation and identification of these products are increasing through the use of refined chromatographic and analytic methods[1,2].

Lichens have long been used in alcohol production and as nourishment in cold countries. They are also used commercially in the perfume, dye and drug industries[3−7]. In addition to their usage as folk medicine for many years, about 60 lichen species are found in different types of antimicrobial, anticancer, antiallergen, immunogical and expectoral preparations[8−11].

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In recent years, especially during summer, the roles of photoactive lichen substances in photosynthesis have been examined in terms of environmental pollution. In addition, many lichen species have been used to measure air pollution by detecting SO_2 and heavy and radioactive metals[12−15].

Some known secondary metabolites of lichens include depsides, depsidones, benzoxasines, benzofuranes, usnic acid and antraquinone derivatives[16−21]. Many scientists have also studied the carbohydrate composition of lichens. Carbohydrates are important in the food and agricultural industries. Polyol linked β-D-glucans are also important in medicine because of their antitumour activities[8,22], and there exists studies about the activity of lichen species on Gram (+) and Gram (−) bacteria and different fungi[23−27].

All of the above properties demonstrate the growing importance of lichens. While there are taxonomic studies available for some Turkish lichens, their chemical compositions have been reviewed in only a limited fashion[21,28].

In this study, the chemical compositions of some Turkish lichens (*Pseudevernia furfuracea*, *Evernia prunastri* and *Letharia vulpina*) were examined by chemical and chromatographic methods and their structures were determined using different spectroscopic techniques.

Experimental

General

The IR spectra of the compounds were recorded as KBr pellets on a Bruker IFS-48 FT-IR spectrometer. UV spectra were recorded by a Jasco V-530 UV-Vis. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were run in $CDCl_3$ on a Bruker-Spectrospin Avance DPX 400 with TMS as internal standard. FAB-mass spectra were carried out on a VG 20-250 quadruple mass spectrometer with Xe bombardment using MeOH as solvent and polyethylene glycol as matrix. EI analyses were also carried out on the same instrument with 70 eV. Merck 7734 and 7747 Silica gels were used for CC and prep. TLC, respectively. TLC was performed on precoated Silica gel 60 (Merck 5554) plates sprayed 10% H_2SO_4 solution. CH_2Cl_2: MeOH solvent systems were used with an increasing polarity beginning from the ratio 9.0:0.1 to MeOH. Antimicrobial activity studies of MeOH extracts were examined by agar-disc diffusion method against *Staphylococcus aureus* ATCC 6538, *Pseudomonas aureoginosa* ATCC 27853 and *Escherichia coli* ATCC 11230 bacteria; *Aspergillus flavus* TEM *Aspergillus oryzae* TEM, and *Aspergillus fumigatus* TEM fungi; and *Candida albicans* ATCC 6538 yeasts. Tobramycine and ketoconazole were used as references for bacteria and yeasts, respectively.

Material

Lichen samples were collected in November, 2000, from Manisa-Spil Mountain (*Pseudevernia furfuracea* (L.) Zoph. and from Kızılcahamam-Ankara (*Evernia prunastri* (L.) Ach., *Letharia vulpine* (L.) Hue). They were deposited in the Herbarium of the Faculty of Pharmacy, Ege University (No. 7435, 7319, 7166, respectively) and identified by one of us (Prof. Dr. Ulvi Zeybek).

Extraction and isolation

Air-dried and powdered *Pseudevernia furfuracea* (1 kg), *Evernia prunastri* (100 g) and *Letharia vulpina* (175 g) were successively extracted with 80% MeOH (3 × 5 L), (3 × 2 L) and (3 × 2 L), respectively. Vacuum dried residues (at 40 °C) were washed with hexane and the CH_2Cl_2. The remaining residues were

dissolved in H_2O and extracted with n-BuOH. Major compounds were observed in CH_2Cl_2 part of the main extract on TLC. The evaporated CH_2Cl_2 layers (9 g) were applied to a Si gel column. The column was eluted with CH_2Cl_2:MeOH solvent systems beginning from the 9.0:0.1 ratio and continued with increasing polarity. After purification, three compounds, Compound **1** (15 mg), Compound **2** (7 mg) and Compound **3** (300 mg), were obtained as amorphous powder.

The structures of these three compounds were analysed using IR, UV, NMR (1D and 2D), EI and FAB-Mass spectra.

Compound 1

A 15 mg amorphous compound was isolated from *Pseudevernia furfuracea*. FT-IR (KBr, cm^{-1}): 3400 (OH), 2935 (Ar-H), 1688 (C=O). UV (CH_2Cl_2, nm): 310, 260, 230. ^{1}H-NMR ($CDCl_3$, 400 MHz, ppm), and ^{13}C-APT ($CDCl_3$, 100 MHz), see Table 1. EI-mass values at m/z: 197$[M+H]^+$ and 164 $[197-(H_2O+CH_3)]^+$ are the molecular ion and main peaks for this molecule in addition to the other fragments at m/z: 136, 108, 79, 76.

Table 1. ^{1}H and ^{13}C NMR data for compound 1.

^{1}H-NMR		^{13}C-NMR	
δ (ppm)	H	δ (ppm)	C
11.96	– COOH	172.99	C-7
6.13	H-5, s, 1 H	163.52	C-4
5.13	Ar-OH, s, 1 H	158.41	C-2
3.85	H-8, s, 3 H	140.55	C-6
2.38	H-9, s, 3 H	110.94	C-5
2.03	H-10, s, 3 H	108.91	C-3
		105.62	C-1
		52.20	C-8
		24.45	C-9
		8.02	C-10

Compound 2

A 7 mg amorphous powder, FT-IR (KBr, cm^{-1}): 3350 (OH), 2925 (Ar-H), 1725 and 1695 (C=O). UV (CH_2Cl_2, nm): 310, 265, 220. ^{1}H-NMR ($CDCl_3$, 400 MHz, ppm), and ^{13}C-APT ($CDCl_3$, 100 MHz), see Table 2. Positive-ion FAB-mass at m/z: 371 $[M+Na]^+$molecular ion, 273 $[M^+-(Na- CH_3- NH_3- CO - CH_3)]^+$ main peak, and at m/z: 333, 316, 288, 259, 165 are the other fragments.

Compound 3

A 300 mg amorphous powder, FT-IR (KBr, cm^{-1}): 3380 (OH), 2954 (Ar-H), 1765, 1680 (C=O). UV (CH_2Cl_2, nm): 310, 265, 220. ^{1}H-NMR $CDCl_3$, 400 MHz, ppm) and ^{13}C-APT ($CDCl_3$, 100 MHz), see Table 3. In EI-mass spectrum molecular ion and main peaks are *m/z*: 322 $[M+H]^+$, and 290 $[M-OCH_3]^+$ respectively. *m/z* 261, 234, 207, 178, 145, 117, 89 are the other fragments of the molecule.

Table 2. ^{1}H and ^{13}C NMR data for compound 2.

^{1}H-NMR		^{13}C-NMR	
δ (ppm)	H	δ (ppm)	C
6.52	H-4', d, 2H	205.96	C-7'
6.44	H-5', d, 2H	176.00	C-7
6.32	H-3, H-5, s, 2H	163.77	C-2
3.79	H-8, s, 3H	144.30	C-6'
3.31	- NH_2, m, 2H	115.45	C-5'
2.61	H-8', s, 3H	111.88	C-5
2.45	H-9, s, 3H	107.83	C-4'
		99.41	C-3
		55.61	C-8
		24.49	C-8'
		23.34	C-9

Table 3. ^{1}H and ^{13}C NMR data for compound 3.

^{1}H-NMR		^{13}C-NMR	
δ (ppm)	H	δ (ppm)	C
13.67	- COOH	172.10	C-1
8.00	H-2', H-6', d, 2H	166.33	C-6
7.28 - 7.13	Ar-H, m, 5H (B ring)	160.78	C-4'
7.10	H-4, s, 1 H	155.20	C-3
7.08	H-3', H-5', d, 2H	131.77	C-1"
3.62	H-7', s, 3 H	129.73	C-2', C-6'
		128.82	C-1'
		128.79	C-4
		128.16	C-2", C-6"
		128.08	C-4"
		127.80	C-3", C-5"
		127.56	C-3', C-5'
		115.63	C-5
		105.43	C-2
		54.89	C-7'

Antibacterial and Antifungal Activities of Lichens

In vitro antimicrobial studies were carried out by agar-disc diffusion methods against different test organisms as mentioned above. Bacteria were inoculated into Muller-Hilton medium (10^{8}cfu/mL) and spore suspension of filamentous fungi (10^{5} spore/mL) were cultured on Sabouraud's dextrose agar with chloramphenicol by plate dilution techniques and Thoma slides, respectively[29,30]. Filamentous fungi and bacteria were added to the medium after autoclaving processes. The MeOH extracts of the materials were placed into the discs in concentrations ranging from 10 to 200 μL MeOH in petri dishes which contained bacteria and yeast. For each experiment another disc that contained only MeOH was used as a control disc. For bacteria and yeast, incubation zone diameters were measured after 24-28 h. Tobramycine and ketoconazole were used as references, under the same conditions as mentioned above, in 10 μg/disc and 70 μg/disc concentrations, respectively.

Results and Discussion

The IR spectrum of compound **1** gave a signal at 1688 cm^{-1}, which indicates the presence of an acidic carbonyl in the molecule in addition to the Ar-H and OH signals. The UV spectrum showed typical aromatic bands confirming a substituted aromatic benzoic acid. In the proton NMR spectrum, the acidic proton's signal was observed at 11.96 ppm. Singlets at 6.13 and 5.13 ppm were assigned to Ar-H and OH protons, respectively. At 3.85 ppm OCH_3 protons were indicated as a singlet. The other two singlets at 2.38 and 2.03 ppm were assigned to two aromatic CH_3 groups. In the ^{13}C-APT spectrum, the signal at 172.99 ppm was assigned to a carboxyl carbon. Six aromatic carbons were observed between 163.52 and 105.62 ppm. A methoxy carbon was determined at 52.20 ppm. CH_3 carbons were detected at 24.45 (C-9) and 8.02 (C-10) ppm. The COSY spectrum of this compound gave obvious homonuclear correlation between Ar-H and CH_3 protons. In the HETCOR spectrum, the heteronuclear H-C correlations for the methyl, methoxy and C-6 carbons with their own protons were observed. The EI-mass spectrum of compound **1** showed a protonated molecular ion peak at m/z 197 (60%) and other fragments confirmed the molecular formula as $C_{10}H_{12}O_4$, named 2-hydroxy-4-methoxy-3,6-dimethyl benzoic acid (Figure 1)[20].

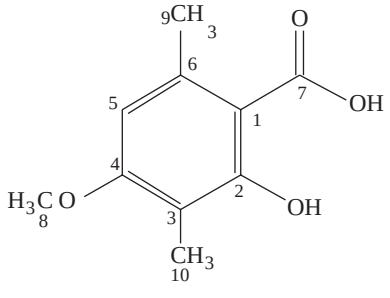

Figure 1.

Compound **2** was obtained as two carbonyl signals at 1725 cm^{-1} and at 1695 cm^{-1} that belonged to ester and ketone carbonyls, respectively. The UV spectrum showed three maxima: 320, 270 and 220 nm. In the proton NMR spectrum, a singlet at 2.45 ppm belonged to CH_3 protons which are bonded to ring A. The methyl protons were observed at 2.61 ppm as a singlet. The multiplet at 3.31 ppm referred to NH_2 protons. The methoxy protons of ring A were observed at 3.79 ppm. Two doublets at 6.52 and 6.44 ppm were attributed to the H-4' and H-5' of ring B, respectively. A singlet at 6.32 ppm belonged to H-3 and H-5 of ring A[20]. The ^{13}C-APT spectrum showed the existence of two CH_3 carbons as the signals at 23.34 ppm for C-9 and at 24.49 ppm for C-8'. The methoxy carbon was observed at 55.61 ppm. The Ar-H carbons 3, 5, 4' and 5' were observed between 115.45 and 99.41 ppm; 144.30 ppm and 163.77 ppm indicated the quaternary carbons C-6' and C-2, respectively. The signals detected at δ 176.00 and 205.96 were indicative for the presence of carbonyl and ketone functionalities, respectively. In the COSY spectrum of this compound a proton-proton correlation was seen between ring A protons and the methyl protons of the same ring. In the HETCOR spectrum of the same compound, heteronuclear C-H correlations were observed between methyl, methoxy, C-3, C-5, C-4' and C-5' carbons with their own protons. The positive-ion FAB-mass spectrum of compound **2** produced a protonated molecular ion peak at m/z: 371$[M+Na]^+$ (15%). The main peak was determined at m/z: 273 $[M^+$-(Na-CH_3-NH_3-CO-CH_3)$]^+$. These peaks and other fragments at m/z: 333, 316, 288, 259 and 165 indicated the molecular formula $C_{17}H_{16}NO_5Cl$, named 3-acetyl-4-amino-2-chloro-1-(4-hydroxy-2-methoxy-6-methylphenylcarbonyloxy)benzene (Figure 2).

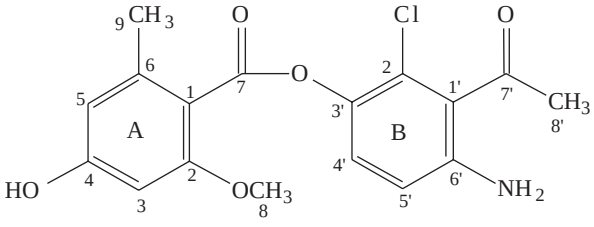

Figure 2.

The IR spectrum of compound **3** showed characteristic hydroxyl (3380 cm^{-1}), lactone (1765 cm^{-1}) and acid (1680 cm^{-1}) carbonyl absorptions. The UV spectrum had three maxima at 380, 280 and 220 nm. In the proton NMR spectrum, the peak at highest field at 3.62 ppm was a methoxy signal. Two doublets at 8.00 and 7.08 ppm concerned the H-2', H-6' and H-3', H-5' of ring A respectively. Other ring protons were observed as a multiplet between 7.28 and 7.13 ppm. A singlet at 7.10 ppm, belonging to H-4, was observed to be very close to the H-3' and H-5' signals. An acidic proton signal was seen in its characteristic region as 13.67 ppm. In the ^{13}C-APT spectrum of the same compound, the methoxy carbon was seen at 54.89 ppm, which was the only signal at high field. Nine aromatic unsubstituted carbons (C-2', C-6', C-3', C-5', C-2", C-6", C-3", C-5", C-4") were seen between 129.73 and 127.56 ppm. Six quaternary carbon signals (C-2, C-3, C-5, C-1', C-4', C-1") were observed between 160.78 and 105.43 ppm. The signal at 128.79 ppm was assigned to C-4. The acid and lactone carbonyl carbons were detected at 172.10 ppm and 166.33 ppm, respectively. The COSY spectrum of this compound showed some specific correlations between H-4 and ring B protons. In the HETCOR spectrum, carbon-proton correlations were observed between methoxy, C-4 and aromatic rings' carbons with their own protons. The protonated molecular ion peak of compound **3** was detected at m/z: 322 (70%) in EI-mass spectrum. This peak and the other fragments at m/z, 290 $[M\text{-}OCH_3]^+$ (main peak), 261, 234, 207, 178, 145, 117 and 89 confirmed the molecular formula as $C_{19}H_{14}O_5$, named 2-(4-methoxyphenyl)-2-(5-oxo-4-phenyl-2,5-dihydro-2-furaniliden) acetic acid (Figure 3).

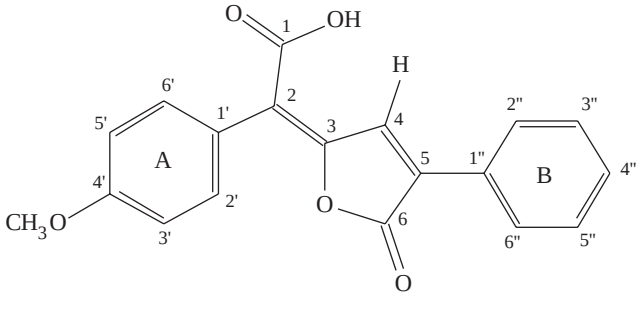

Figure 3.

The microbiological studies of MeOH extracts of *Pseudevernia furfuracea*, *Evernia prunastri* and *Letharia vulpina* showed measurable activity at all concentrations against the bacterium *Staphylococcus aureus* between 22.5 and 25.0 mm zone diameters. For fungal examinations, the inhibitions of *Evernia prunastri* and *Letharia vulpina* were observed against *Aspergillus fumigatus* at the zone diameters 20.0-22.5 mm. When these values were compared with tobramycine (10.0-16.0 mm), it is seen that the zone diameters of the bacteria were larger than the zone diameter of reference. The zone diameters of *Aspergillus fumigatus* were smaller than the zone diameter of ketoconazole (28.0-35.0 mm); the MeOH extracts are barely active

against the yeast used for the measured values (Table 4). All these results confirm the importance of the above isolated and structurally determined natural products. This is especially so, when one considers the great importance of drugs obtained from different lichen species that are used against *Staphylococcus aureus.*

Table 4. The activity results of the MeOH extracts

Lichens / Bacteria	*Evernia prunastri*	*Letharia vulpine*	*Pseudoevernia furfurecea*	*Tobramycine* (10 μg/disc)	*Ketoconazole* (70 μg/disc)
Staphylococcus aureus ATCC 6538	25.0	22.5	22.9	16	n.t.
Pseudomonas aureoginosa ATCC 27853	-	-	-	12	n.t.
Escherichia coli ATCC 11230	-	-	-	10	n.t.
Yeast					
Candida albicans ATCC 6538	-	-	-	n.t.	n.t.
Fungi					
Aspergillus flavus TEM	22.5	20.0	-	n.t.	32
Aspergillus oryzae TEM	-	-	-	n.t.	35
Aspergillus fumigatus TEM	-	-	-	n.t.	28

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