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Antimicrobial Metabolites from Three Serbian *Caloplaca*

Nedeljko T. Manojlovic, Milan Novakovic, Vladeta Stevovic, and Slavica Solujic

Faculty of Science, University of Kragujevac, Serbia and Montenegro

Abstract

Methanol extracts of three *Caloplaca* species showed broad-spectrum antifungal and antibacterial properties against some human and plant pathogens. Fractionation with methylene chloride and isolation of pure anthraquinone derivatives substantially increased the level of antimicrobial activity. Eight anthraquinone derivatives were isolated from the three species. 1-*O*-Methylparietin and 8-*O*-methylparietin were isolated from lichens for the first time. Minimum inhibitory concentrations for the extracts were found to be 80–320 μg/ml for bacteria and 40–320 μg/ml for fungi, and the pure anthraquinones range from 20 to 320 μg/ml for bacteria and from 20 to 160 μg/ml for fungi.

Keywords: Anthraquinones, antibacterial activity, antifungal activity, *Caloplaca*.

Introduction

Lichens produce a diverse range of primary and secondary metabolites (Hale, 1983). Slow growth, and often harsh living conditions, makes production of protective compounds a necessity for lichens. The secondary metabolites possess a broad spectrum of biological activities including antibacterial (Lauterwein et al., 1995; Yamamoto et al., 1998), enzyme inhibition (Matsubara et al., 1997), antifungal (Proksa et al., 1996; Shahi et al., 2001), analgesic and antipyretic (Okuyama et al., 1994), antitumor (Kupchan & Kopperman, 1975), and antiviral (Cohen et al., 1996) activity.

Caloplaca lactea, *C. citrina*, and *C. schaereri* are lichens belonging to the family Teloschistaceae. Previously, only four different anthraquinone derivatives from these three lichen species have been detected by thin-layer chromatography (TLC) and lichen mass spectrometry (LMS) but not isolated (Santesson, 1970).

Anthraquinones are components in many medicines of plant origin because they possess antibacterial, anti inflammatory, antitumor, purgative, astringent, antiviral (Cyong et al., 1987; Muzychkina, 1998), and antifungal properties (Agarwal et al,. 2000; Manojlovic et al., 2001). Lichens produce some characteristic anthraquinone derivatives, which have yet to be found in higher plants (Santesson, 1970; Nakano et al., 1972; Steglich & Reininger, 1972; Søchting, 1997, 2001; Rosso et al., 2003).

Materials and Methods

Lichen *species* studied

The lichens of *Caloplaca lactea* (Massal.) Zahlbr. and *C. citrina* (Hoffm.) Th.Fr. were collected in July 2000 from Gledic Mountain (Serbia and Montenegro), and *C. schaereri* (Arnold) Zahlbr. was collected in July 1998 from Durmitor Mountain (Serbia and Montenegro). These *Caloplaca* species were identified by S. Savic, Natural History Museum, Belgrade, Serbia and Montenegro, where voucher specimens have been deposited (*C. lactea*, BEO 1446; *C. citrina*, BEO 1444; *C. schaereri*, 1445).

General experimental procedures

UV-Vis spectra were recorded on a Perkin Elmer Lambda 35 UV-Vis spectrometer, using ethanol as the solvent, and mass spectra was determined with a Finigan-MAT 8230 instrument. ^{1}H NMR spectra were recorded on a Varian Gemini (200 MHz) instrument in $CDCl_3$ using TMS as an internal standard. Melting points (m.p.) were determined on a Kofler hot-stage apparatus and are uncorrected. Silica gel (Merck,

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Address correspondence to: Prof. Dr. Nedeljko T. Manojlovic, Faculty of Science, University of Kragujevac, P.O. Box 60, 34000 Kragujevac, Serbia and Montenegro. E-mail: mtnedeljko@yahoo.com

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0.063–0.02 mm) and Sephadex LH-20 were used for column chromatography. Preparative layer chromatography was performed on 20 × 20 cm glass plates coated with 1 mm of silica (Merck, Kieselgel GF_{254} applied as a suspension in H_2O).

Preparation of lichen extracts and active principles

Methanol extracts of three lichen species were prepared in a Soxhlet apparatus using 20 g of each lichen in 200 ml solvent for 6 h. These extracts were used for antimicrobial and chemical determination. The extracts were analyzed by TLC on silica gel using benzene and benzene-acetone mixtures as eluent, detected under UV 254 nm, and visualized by spraying with a methanolic solution of magnesium acetate along with standard markers (parietin, fallacinal, fallacinol, emodin, erythroglaucin, and parietinic acid). The methanol extracts were fractionated with methylene chloride, and after TLC determination metabolites from the methylene chloride fractions were separated on a silica gel column using benzene, benzene-acetone (20:1, 10:1, and 5:1), and acetone as eluents. Finally, the column was washed with a mixture of ethyl acetate/methanol/acetone. The results of chemical analysis are shown in Table 1. Eight structurally different multisubstituted anthraquinone derivatives were isolated (Fig. 1).

Spectral data for new lichen metabolites

8-*O*-Methylparietin: yellow needles; m.p. 192–194°C; UV-Vis (ethanol): ($\lambda \log \varepsilon$): 223 (3.96), 268 (4.55), 285 (4.60) and 428 (3.86). Mass spectrum m/z (%): 298 (M^+, 100 %), 280 (39), 269 (20), 252 (44), 149 (75). ^{1}H NMR ($CDCl_3$): δ: 2.51 (3H, s, Me), 3.92 (3H, s, OMe), 4.06 (3H, s, OMe), 6.71 (1H, d, *J*-2.5 Hz, H-2), 7.12 (1H, br s, H-7), 7.31 (1H, d, *J*-2.5 Hz, H-4), 7.77 (1H, br s, H-5), and 13.32 (1H, s, OH-1).

1-*O*-Methylparietin: yellow needles; m.p. 212–213°C; UV-Vis (ethanol): ($\lambda \log \varepsilon$) 270 (4.18), 282 (4.20) and 426 (3.85). Mass spectrum m/z (%): 298 (M^+) (100), 281 (70), 280 (97), 269 (84), 252 (78). ^{1}H NMR ($CDCl_3$): δ 2.44 (3H, s, Me), 3.99 (3H, s, OMe), 4.04 (3H, s, OMe), 6.80 (1H, d, *J*-2.5 Hz, H-2), 7.08 (br s, 1H, H-7), 7.48 (d, *J*-2.5 Hz, 1H, H-4), 7.58 (1H, br s, H-5), and 13.11 (1H, s, OH-8).

Table 1. Distribution of anthraquinone derivatives in three *Caloplaca*.

Anthraquinones	*C. lactea*	*C. citrina*	*C. schaereri*
Erythroglaucin	+	−	−
Parietin[a]	+	+	+
Fallacinal	+	+	+
Fallacinol	+	+	+
1-*O*-Methylparietin	+	−	−
8-*O*-Methylparietin	+	−	−
Emodin	+	+	+
Parietinic acid	−	−	+

[a]The most abundant anthraquinone in all lichen tested.

Microorganisms

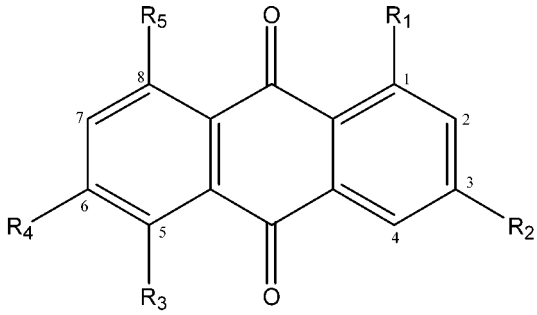
The bacterial strains of *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* and fungal strains of *Candida albicans*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Trichoderma harzianum* were used for the antimicrobial investigations. All fungi and bacteria were obtained from the culture collection maintained in the Department of Biology, Faculty of Science, University of Kragujevac, Serbia and Montenegro.

Antimicrobial assays

Antimicrobial activity *in vitro* was studied by determination of the minimum inhibitory concentrations (MICs) using a twofold broth dilution method as previously described in *Lichenologist* (Manojlovic et al., 2002). Anthraquinones and lichen extracts at concentrations 10, 20, 40, 80, 160, and 320 μg/ml were used. Inoculation was carried out with overnight cultures of the strains, to a final inoculum size of 10^5 CFU/ml for the bacteria and 10^4 CFU/ml for the fungi. MICs were read after 24 h incubation at 37°C for the bacteria and 48 h at 30°C for the fungi and were taken equal to the lowest concentration of the compounds where no visually observable growth.

Results and Discussion

Air-dried *Caloplaca lactea*, *C. citrina*, and *C. schaereri* were extracted with methanol, and all the extracts (ME) obtained were tested for antibacterial and antifungal activity. The lichen extracts exerted varying levels of antimicrobial effects against microorganisms. MIC values of the tested extracts are shown in Table 2. MIC values for bacteria are from 160 to 320 μg/ml. *C. lactea* methanol extract showed greater antimicrobial effects against all microbial species than other lichen methanol extracts. On the other hand, all the tested extracts did not show antibacterial effects on Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* (MIC > 320 μg/ml). Testing of the methanol extracts on four fungi showed the MICs to be 80–320 μg/ml. Fractionation with methylene chloride increased the level of activity considerably. In this testing, the Gram-positive bacteria were more sensitive than Gram-negative bacteria, and the MIC values for them ranged from 80 to 320 μg/ml depending on the extract. The MIC values for fungi were 40–320 μg/ml. The results



		R_1	R_2	R_3	R_4	R_5
1	Erythroglaucin	OH	OCH_3	OH	CH_3	OH
2	Parietin	OH	OCH_3	H	CH_3	OH
3	Fallacinal	OH	OCH_3	H	CHO	OH
4	Fallacinol	OH	OCH_3	H	CH_2OH	OH
5	1-*O*-Methylparietin	OCH_3	OCH_3	H	CH_3	OH
6	8-*O*-Methylparietin	OH	OCH_3	H	CH_3	OCH_3
7	Emodin	OH	OCH_3	H	OH	OH
8	Parietinic acid	OH	OCH_3	H	COOH	OH

Figure 1. Structures of isolated compounds.

Table 2. Minimum inhibitory concentration (MIC) values (μg/ml) for antibacterial and antifungal activity of the methylene chloride (MH) and methylene (ME) extracts of *C. lactea*, *C. citrina*, and *C. schaereri*.

	MIC[a] (μg/ml)					
	C. lactea		*C. citrina*		*C. schaereri*	
	ME	MH	ME	MH	ME	MH
Bacteria						
B. cereus	160	80	320	160	320	160
B. subtilis	160	180	>320	320	320	160
S. aureus	320	160	>320	320	>320	320
E. coli	>320	>320	>320	>320	>320	>320
P. aeruginosa	>320	>320	>320	>320	>320	>320
Fungi						
T. mentagrophytes	160	80	320	160	320	160
E. flocossum	>320	160	>320	320	>320	320
C. albicans	320	160	320	160	>320	320
T. harzianum	80	40	160	80	80	40

[a]Values with the sign ">" indicate that the highest concentrations tested did not inhibit the bacterial or fungal growth.

showed that methylene chloride fractions of *C. lactea* and *C. schaereri* exhibited the greatest activity toward *Trichoderma harzianum* (MIC 40 μg/ml). As in the case of methanol extracts, methyene chloride fractions were not effective on Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* even at higher concentrations (MIC > 320 μg/ml).

After examining the antibacterial and antifungal activity of the extracts, secondary anthraquinone metabolites were isolated and identified from the extracts. These were suspected to be carriers of exhibited activity. Methylene chloride fractions contain the same anthraquinone derivatives as well as methanol extracts of the corresponding lichen. Methanol extracts contained polyols (mainly D-mannitol and D-arabitol) besides anthraquinones and some other minor compounds, whereas in the methylene chloride fractions polyols could not be found. Anthraquinones were separated on a silica gel column using benzene, benzene-acetone (20:1, 10:1, and 5:1), and acetone as eluents. Four anthraquinone derivatives from the *C. citrina* extract—parietin, fallacinal, fallacinol, and emodin—were isolated. Parietin was

the primary constituent of the three extracts. Chromatographic purification of the methylene chloride extract of *C. schaereri* produced parietin, fallacinal, fallacinol, emodin, and parietinic acid. Their identities were established by comparison of the m.p. UV-Vis, ^{1}H NMR, and mass spectral data with that of the known compounds (Krivoshchekova et al., 1981; Yoshimura et al., 1994; Manojlovic et al., 2001; Rosso et al., 2003). Chemical analysis of *C. lactea*, whose extract exhibited the most significant level of antibacterial and antifungal activity, showed anthraquinone derivatives not found in *C. citrina* and *C. schaereri*. Seven anthraquinone derivatives were identified in the methylene chloride extract using column and preparative layer chromatography with different eluents. Besides parietin, fallacinal, fallacinol, emodin, and eryhroglaucin, two *O*-alkylated parietin derivatives (not previously found in *Caloplaca*) were isolated from the *C. lactea* extract. The identities of these compounds were established by m.p., ^{1}H NMR, UV and mass spectra. This is the first report of parietin ethers that could have been formed by methylation of hydroxyl groups of parietin (at C-1 and C-8), similar to methylation of the hydroxyl group of emodin (at C-6). These anthraquinones were synthesized earlier from emodin derivatives (Savard & Brassard, 1984) and cycloaddition of diene and napthaquinones (Cameron & Crossley, 1977).

After isolation and identification, the pure anthraquinones were tested on the same species of bacteria and fungi, as in the case of the extracts. The results showed a more significant level of antimicrobial activity. The anthraquinones exhibited considerable antibacterial effects on the species tested with MICs ranging from 20 to 320 μg/ml. Fallacinol showed the best effects against-*Staphylococcus aureus* (MIC 20 μg/ml) and *Bacillus subtilis* (MIC 40 μg/ml), from which it may be concluded that an anthraquinone with a hydroxymethyl group in position C-3 shows greater antibacterial activity than other derivatives. 1-*O*-methyl parietin and 8-*O*-methyl parietin showed antibacterial effect toward Gram-positive species *Bacillus cereus*, *Bacillus subtilis*, and *Staphylococcus aureus* with MIC 40–160 μg/ml.

None of the compounds tested showed inhibitory activity toward Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* (MIC > 320 μg/ml). Examination of the results of the antifungal activity showed significant inhibition against *Candida albicans*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, and *Trichoderma harzianum*. These anthraquinones demonstrated greater antifungal activity than crude extracts and metabolites of some anthraquinone-containing medical plants (Agarwal et al., 2000). As shown in Table 3, MICs were 10–160 μg/ml. Among the tested compounds, fallacinol, which is identified as a constituent of all three species examined, showed the most potent antifungal effect on *Trichoderma harzianum* (MIC 10 μg/ml).

These results show that the anthraquinone structure plays a significant role in expressing antibacterial and antifungal activity, but the presence of both hydroxyl groups (at C-1 and C-8) is not necessary for exhibiting this activity. In the light of the above, it is concluded that *C. lactea*, whose extract exhibits the highest level of activity, is chemically different from the other *Caloplaca* that were investigated (Santesson, 1970; Søchting, 1997, 2001) and contains the greatest number of structurally different anthraquinones. The *O*-alkylated parietin derivatives also show significant antimicrobial activity, which confirms the hypothesis that lichens represent an important source for the production of new antimicrobial agents.

Table 3. Minimum inhibitory concentration (MIC) values (μg/ml) for antibacterial and antifungal activity of the anthraquinones from *C. lactea*, *C. citrina*, and *C. schaereri*.

	MIC[a] (μg/ml)							
	1	2	3	4	5	6	7	8
Bacteria								
B. cereus	80	80	40	40	80	40	80	40
B. subtilis	80	80	80	40	80	40	80	40
S. aureus	320	320	160	160	160	20	320	80
E. coli	>320	>320	>320	>320	>320	>320	>320	160
P. aeruginosa	>320	>320	>320	>320	>320	>320	>320	>320
Fungi								
T. mentagrophytes	40	20	20	40	20	20	40	40
E. flocossum	160	160	160	160	160	160	160	160
C. albicans	80	80	80	80	80	80	80	80
T. harzianum	40	20	40	40	20	10	40	20

1, erythroglaucin; 2, parietin; 3, fallacinal; 4, fallacinol; 5, 1-*O*-methylparietin; 6, 8-*O*-methylparietin; 7, emodin; 8, parietinic acid.
[a]Values with the sign ">" indicate that the highest concentrations tested did not inhibit the bacterial or fungal growth.

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