

Allelopathic effects of lichen secondary metabolites on lichen photobiont *Trebouxia erici*

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ABSTRACT

Atranorin, gyrophoric acid and parietin were isolated from the lichens: *Psudevernia furfuracea*, *Umbilicaria hirsuta* and *Xanthoria parietina* to compare their phytotoxicity on cultures of aposymbiotically grown lichen photobiont *Trebouxia erici*. The cortical didepside atranorin had strong phytotoxic effects on the photobiont cells. Application of atranorin at the highest dose (0.1mg/disk) decreased the growth of photobiont cells and altered the composition of assimilation pigments (decreased chlorophyll *a* content, its fluorescence and increased its phaeophytinization). Atranorin also induced the formation of reactive oxygen species, hydrogen peroxide and superoxide. Atranorin as cortical lichen compound, may be in direct contact with the photobiont cells forming layer in lichens with a stratified thallus. Thus it appears that atranorin may be functioning as an allelochemical, controlling the cell division of algal partner inside the thallus and thereby regulating the balance between the symbionts. However, phytotoxicity of tridepside gyrophoric acid and anthraquinone parietin on the photobiont cells was not observed at the tested concentrations used in this study.

Key words: Algae, allelochemicals, lichens, *Psudevernia furfuracea*, secondary metabolites, toxicity, *Trebouxia erici*, *Umbilicaria hirsuta*, *Xanthoria parietina*

INTRODUCTION

Lichens [symbiotic association between fungi (mycobionts) and algae and/or cyanobacteria (photobionts, phycobionts)] are important components of vegetation worldwide. They produce unique extracellular secondary metabolites called lichen substances (12), which have many ecological roles [antimicrobial, antiherbivoral and allelopathic activities (8,10,14,16)]. Besides these substances can be involved in protection of photobiont cells from excessive light and making chelates with potentially toxic metals (13,17). The crystals of cortical lichen secondary compounds may be deposited directly on the surface of photobiont cells (15,18), hence, these compounds may be phytotoxic to the algal partner in lichen thalli. Usnic acid, present in many lichen species and most studied lichen secondary metabolite, may regulate the photobiont population in the thallus by inhibiting the photobiont cell division (5). As lichen photobionts grow very slowly, the

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production of secondary metabolites may be a critical step in maintaining the required ratio between the symbionts.

As only the influence of usnic acid on selected physiological parameters and growth of lichen photobiont (Genus *Trebouxia*) have been tested (5), hence, it is difficult to broadly consider the phytotoxic effects of lichen compounds on photobiont cells. We hypothesize that lichen photobionts are differentially sensitive to secondary metabolites from various chemical classes. To test this hypothesis, we isolated didepside atranorin, tridepside gyrophoric acid and anthraquinone parietin from lichens containing a photobiont from the genus *Trebouxia* (*sensu lato*) and evaluated their potential phytotoxic effects on aposymbiotically grown lichen photobiont *Trebouxia erici*.

MATERIALS AND METHODS

I. Lichens and secondary metabolites

Didepside atranorin (CAS no.: 479-20-9) was isolated from the lichen *Pseudevernia furfuracea* collected from the stems of *Pinus sylvestris* near Harmanec, central Slovakia. Tridepside gyrophoric acid (CAS no.: 548-89-0) was isolated from the lichen *Umbilicaria hirsuta* collected from the rocks near Harmanec, central Slovakia. Lichens were identified by Prof. Martin Bačkor. Voucher specimens of each lichen species were stored in our laboratory for future reference. Anthraquinone parietin (CAS no.: 521-61-9) was isolated previously in our laboratory (6) and purity was assessed by TLC and HPLC (approximately 98 %).

Secondary metabolites were isolated from the lichens as per protocols of Asahina and Shibata (3) and Solhaug and Gauslaa (16). Crystals of compounds were finally dissolved in pure acetone and re-crystallized. Purity of compounds was assessed using TLC and HPLC (6). Mean contents of secondary metabolites in lichens were: atranorin (5.6%), gyrophoric acid (6.2%) and parietin (3.4%).

Electrospray mass spectra (ESI-MS) were recorded on a LCQ Finnigan Mass Spectrometer. ESI-HR mass spectra were recorded on APEX IV, 7T, FT-ICR MS Bruker Daltonik. Electron Impact Mass-spectra (EI-HRMS) were recorded on a double focusing sector field mass spectrometer AMD-402 (AMD Inectra, Harpstedt, Germany). ^1H (300 and 600 MHz) and ^{13}C (75.5 and 125.7 MHz) NMR spectra were measured on a Bruker AMX 300 and on a Varian Inova 600 (599.740 MHz) spectrometer in DMSO- d_6 . The chemical shifts are expressed in δ values with TMS as an internal standard. ^1H - ^1H COSY, ^1H - ^1H NOESY, HSQC and HMBC were obtained by conventional methods. UV-vis spectroscopy was recorded on a Specord 2000 instrument (Analytik Jena, Germany). IR spectra were recorded on a Beckman DU 601 and Shimadzu IR scanning spectrophotometers.

II. Organisms and culture conditions

Aposymbiotically grown lichen photobiont *Trebouxia erici* Ahmadjian (UTEX 911) was used in this study. Photobiont cultures were cultivated in solid *Trebouxia* medium (1), pH 6.5 and grown at 22°C under 16-h photoperiod and 30 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ artificial irradiance ("cool white" tubes).

III. Toxicity assay

Algae were cultivated on the surface of Whatman CF/C filters (glass fiber filter disks, 25 mm dia). Filters were subjected to different pretreatments. Atranorin, gyrophoric acid and parietin (0.01 and 0.1 mg / disk) were dissolved in acetone (volume 30 μ l) and applied by automatic pipette on the surface of disks, while the same volume of acetone was used for control disks. The toxicity assay has been described in detail (5). The total mass of cultures was calculated by subtracting the mean dry weight (dw) of a Whatman CF/C disk saturated by identical medium, from the dw of a disk supporting algal cultures 14 days after cultivation. Each treatment was replicated eighteen times.

The assimilation pigments, chlorophyll *a* fluorescence, content of soluble proteins and oxidative status (content of hydrogen peroxide and superoxide) were analyzed as described previously (5).

Statistical analysis: The one-way analysis of variance and Tukey's pairwise comparisons (MINITAB Release 11, 1996) were applied to determine the significance ($P < 0.05$) of differences in all measured parameters.

RESULTS AND DISCUSSION

Identity and purity of lichen secondary metabolites

Purification of acetone extract of *Pseudevernia furfuracea* afforded compound namely atranorin (12). Atranorin was isolated as a white powder, it was soluble in lower alcohols, dimethylsulfoxide, N,N-dimethylformamide, acetone, ethyl acetate and chloroform, but insoluble in water and *n*-hexane. It gave a blue color reaction with a 0.5% solution of vanillin in methanol/sulfuric acid/acetic acid and FeCl₃ reagents, but not with ninhydrin and Sakaguchi reagents. Atranorin showed strong absorption in its UV-vis spectrum at 305, 265 and 250 nm. The structure of atranorin was settled on the basis of electrospray (ESI-MS), high-resolution electron impact mass spectrometry (EI-HRMS) and extensive NMR studies (¹H, ¹³C, APT, ¹H-¹H COSY, HSQC and HMBC). In the ¹³C NMR and APT spectra of atranorin all 19 carbon signals were visible (Table 1), which were assignable to two carbonyl ester groups at δ_C 157.5 and 169.5, twelve aromatic carbons (2 CH and 10 Cq signals), three aromatic methyl carbons (δ_C 9.2, 18.7 and 21.2) and one methoxy carbon at δ_C 52.2. It also showed a carbon aldehyde group at δ_C 193.5. The chemical shifts of all proton and carbon atoms of atranorin are summarized in Table 1.

Gyrophoric acid (GA) was isolated from the lichen *Umbilicaria hirsuta* (12). Gyrophoric acid was purified by solvent extraction, silica gel column chromatography and preparative HPLC consecutively. GA was obtained as a colorless plate crystals, which was soluble in lower alcohols, dimethylsulfoxide, N,N-dimethylformamide and ethyl acetate, but insoluble in water, ether and *n*-hexane. It gave a yellow color reaction with a 3% solution of vanillin in methanol/sulfuric acid and FeCl₃ reagents, but not with ninhydrin and Sakaguchi reagents. The structure of gyrophoric acid was settled on the basis of electrospray (ESI-MS), high-resolution electron impact mass spectrometry (EI-HRMS) and extensive NMR studies (¹H, ¹³C, APT, ¹H-¹H COSY, HSQC and HMBC). The (-)-ESI mass spectrum of gyrophoric acid displayed *pseudo*-molecular ion at *m/z* 467.1

Table 1. ^1H and ^{13}C NMR chemical shifts of atranorin (**1**) in $\text{DMSO-}d_6$ (600 MHz and 125 MHz, TMS as internal standard, chemical shifts in δ values)

Assignment	δ_{H}	HMBC	H \rightarrow C	δ_{C}	Groups
1	6.42 (1H, s)	C-2, C-3, C-5, C-8		108.9 d	CH=
2	-	-		163.2 s	Cq
2-OH	10.55 (1H, s, br)	C-2		-	
3	-	-		110.5 s	Cq
4	-	-		164.3 s	Cq
4-OH	10.55 (1H, s, br)	C-4		-	
5	-	-		107.8 s	Cq
6	-	-		151.2 s	Cq
7	-	-		157.5 s	C=O
8	2.48 (3H, s)	C-1, C-5, C-6		21.2 q	CH3
9	10.27 (1H, s)	C-3, C-4		193.5 d	HC=O
1'	-	-		148.8 s	Cq
2'	6.65 (1H, s)	C-1', C-4', C-9'		115.5 d	CH=
3'	-	-		136.4 s	Cq
4'	-	-		116.1 s	Cq
5'	-	-		161.4 s	Cq
5'-OH	10.55 (1H, s, br)	C-5'		-	
6'	-	-		114.8 s	Cq
7'	-	-		169.5 s	C=O
8'	3.90 (3H, s)	C-7'		52.2 q	O-CH3
9'	2.41 (3H, s)	C-2, C-3', C-4'		18.7 q	CH3
10'	2.38 (3H, s)	C-1', C-5', C-6'		9.2 q	CH3

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

(M-H) $^-$. The molecular mass (468 Da) and the chemical formula $\text{C}_{24}\text{H}_{20}\text{O}_{10}$ were readily determined by high-resolution electron impact mass spectrometry. This formula indicated 15 double bond equivalents.

In the ^1H NMR spectrum ($\text{DMSO-}d_6$) of GA, the low-field signals at δ_{H} 6.23, 6.25, 6.61, 6.63, 6.65 and 6.70 were peculiar to six aromatic protons. Furthermore, the protons of three methyl groups were observed at δ_{H} 2.38, 2.38 and 2.40. The hydroxyl proton resonances occur by δ_{H} 10.47 and 10.32. The chemical shifts of all proton and carbon atoms of GA are summarized in Table 2.

Toxicity assay

Biomass production in *Trebouxia erici* decreased in response to increased atranorin doses. At the highest external dose (0.1 mg/disk), the biomass production of photobiont decreased -85.9% when compared with respective control cultures (Table 3; Fig. 1A). Presence of gyrophoric acid and parietin had no significant effects on the growth of photobiont cultures.

Assimilation pigment composition (chlorophyll *a*, chlorophyll *a+b*, total carotenoids) was significantly decreased by the highest (0.1 mg/disk) tested dose of atranorin (Table 4, -73.7%, -31.1% and -54.1% when compared with respective control cultures; Figs. 2A, 2C, 2D), while chlorophyll *b* content did not change significantly when

Table 2. ^1H and ^{13}C NMR chemical shifts of gyrophoric acid (**2**) in $\text{DMSO-}d_6$ (600 MHz and 125 MHz, TMS as internal standard, chemical shifts in δ values)

Assignment	δ_{H}	HMBC	H \rightarrow C	δ_{C}	Groups
1	6.23 (1H, s)	C-2, C-3, C-5		110.1 d	CH=
2	-	-		166.9 s	Cq
2-OH	10.47 (1H, s, br)	-		-	
3	6.25 (1H, s)	C-5		100.2 d	CH=
4	-	-		165.5 s	Cq
4-OH	10.47 (1H, s, br)	-		-	
5	-	-		105.0 s	Cq
6	-	-		141.0 s	Cq
7	-	-		170.1 s	C=O
8	2.38 (3H, s)	C-6		21.7 q	CH ₃
1'	-	-		156.0 s	Cq
2'	6.63 (1H, s)	C-1', C-4', C-8'		114.2 d	CH=
3'	-	-		139.9 s	Cq
4'	-	-		117.0 s	Cq
5'	-	-		160.0 s	Cq
5'-OH	10.32 (1H, s, br)	-		-	
6'	6.70 (1H, s)	C-1', C-4'		107.2 d	CH=
7'	-	-		170.1 s	C=O
8'	2.40 (3H, s)	C-2', C-3'		21.0 q	CH ₃
1''	-	-		152.1 s	Cq
2''	6.65 (1H, s)	C-3'', C-4''		107.0 d	CH=
3''	-	-		159.1 s	Cq
3''-OH	10.32 (1H, s, br)	-		-	
4''	-	-		118.0 s	Cq
5''	-	-		138.0 s	Cq
6''	6.61 (1H, s)	C-4'', C-1''		115.0 d	CH=
7''	-	-		172.9 s	C=O
8''	2.38 (3H, s)	C-5''		19.1 q	CH ₃

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

Table 3. Biomass production (mg dw/disc), chlorophyll *a* integrity (A 435/A 415 nm), chlorophyll *a/b* ratio (A/B) and chlorophyll *a* fluorescence (F_V/F_M) of 2 weeks old *Trebouxia* cultures cultivated on disks with addition of atranorin, gyrophoric acid and parietin (0, 0.01 and 0.1 mg).

Metabolite	Concentration (mg/disk)	Biomass (mg DW/disc)	A 435/A 415	A/B	F_V/F_M
	0	11.8 \pm 1.6a	1.39 \pm 0.04a	2.89 \pm 0.20a	0.62 \pm 0.06a
Atranorin	0.01	4.9 \pm 0.7b	1.30 \pm 0.06a	2.42 \pm 0.04a	0.55 \pm 0.01a
	0.1	1.7 \pm 0.5c	0.94 \pm 0.11b	1.23 \pm 0.27b	0.24 \pm 0.03b
Gyrophoric a.	0.01	9.9 \pm 1.3a	1.37 \pm 0.05a	3.03 \pm 0.15a	0.60 \pm 0.02a
	0.1	10.3 \pm 0.8a	1.34 \pm 0.06a	3.00 \pm 0.52a	0.57 \pm 0.03a
Parietin	0.01	9.1 \pm 2.6a	1.36 \pm 0.02a	3.26 \pm 0.76a	0.59 \pm 0.02a
	0.1	9.8 \pm 1.9a	1.32 \pm 0.06a	3.35 \pm 0.23a	0.61 \pm 0.03a
	F value	F = 15.82	F = 19.13	F = 10.21	F = 52.46
	P value	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

Table 4. Chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b* and total carotenoids contents of 2 weeks old *Trebouxia* cultures cultivated on disks with atranorin, gyrophoric acid and parietin (0, 0.01 and 0.1 mg).

Metabolite	Concentration (mg/disk)	Chlorophyll <i>a</i> (mg/g DW)	Chlorophyll <i>b</i> (mg/g DW)	Chlorophyll <i>a+b</i> (mg/g DW)	Carotenoids (mg/g DW)
Atranorin	0	2.55 ± 0.36a	0.83 ± 0.16a	3.38 ± 0.41a	0.83 ± 0.16a
	0.01	2.90 ± 0.47a	1.08 ± 0.21a	4.04 ± 0.67a	0.98 ± 0.15a
	0.1	0.66 ± 0.19b	0.57 ± 0.24a	1.24 ± 0.43b	0.38 ± 0.19b
Gyrophoric a.	0.01	2.88 ± 0.36a	0.93 ± 0.15a	3.81 ± 0.46a	0.77 ± 0.10a
	0.1	2.54 ± 0.36a	0.83 ± 0.05a	3.42 ± 0.31a	0.83 ± 0.05a
Parietin	0.01	2.98 ± 0.50a	0.96 ± 0.40a	3.93 ± 0.76a	1.11 ± 0.25a
	0.1	2.88 ± 0.85a	0.89 ± 0.25a	3.83 ± 1.09a	0.94 ± 0.30a
	F value	F = 7.63	F = 1.74	F = 5.62	F = 3.54
	P value	P = 0.001	P = 0.158	P = 0.004	P = 0.018

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

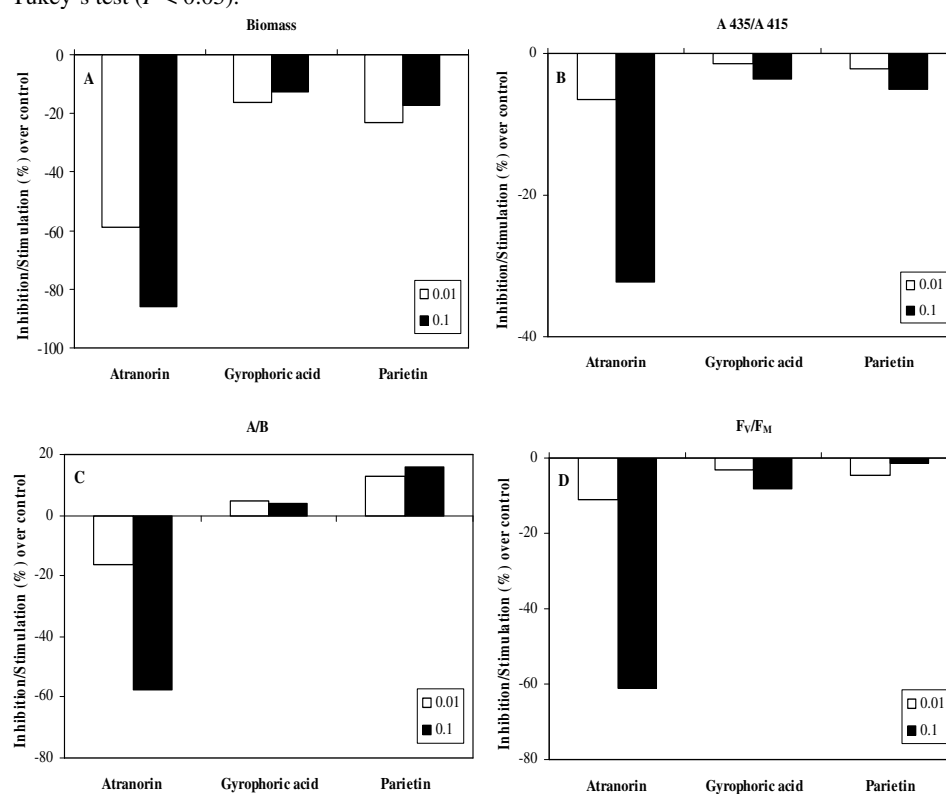


Figure 1. Effects of lichen secondary metabolites on selected physiological parameters of 2 weeks old *Trebouxia* cultures (A: Biomass production, B: Chlorophyll *a* integrity, C: Chlorophyll *a/b* ratio, D: Chlorophyll *a* fluorescence).

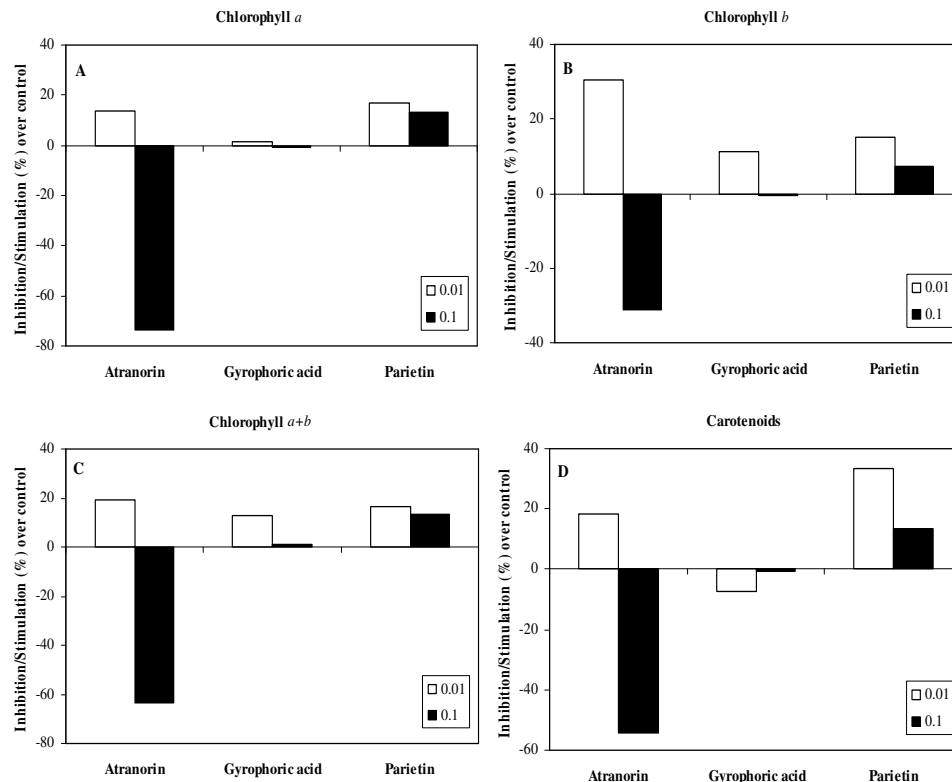


Figure 2. Effects of lichen secondary metabolites on selected physiological parameters of 2 weeks old *Trebouxia* cultures (A: Chlorophyll *a* content, B: Chlorophyll *b* content, C: Chlorophyll *a+b* content, D: Total carotenoids content).

calculated for unit of dry weight (Fig. 2B). The highest tested dose of atranorin decreased chlorophyll *a* integrity (A 435/A 415 nm, which reflects the ratio of chlorophyll *a* to phaeophytin *a*) and chlorophyll *a/b* ratio (Table 3, -32.3% and -57.4% when compared with respective control cultures; Figs. 1B, 1C). Chlorophyll *a* fluorescence decreased in photobiont cultures due to the highest tested dose of atranorin (Table 3, -61.2% when compared with respective control cultures; Fig. 1D). Presence of gyrophoric acid and parietin had no significant effects on the measured photosynthetic parameters of photobiont cultures. Similarly, as was found in previous studies focused on chlorophyll composition of plants exposed to xenobiotics, lichen photobionts responded to environmental stress by decreasing levels of chlorophyll *a* rather than chlorophyll *b* (4). It has been demonstrated that atranorin action on photobiont cells is similar to the effect of usnic acid, as degradation of chlorophyll *a* was also accompanied by increased phaeophytinization of chlorophyll *a* (5). Takahagi et al. (18) found that PSII in the cells of lichen photobionts are relatively protected against the phytotoxicity of lichen secondary compounds. In the present study it was found true for the effects of gyrophoric acid and

parietin, while the effect of atranorin on PSII of the photobiont cells was cytotoxic. Bačkorová et al. (6) observed differential cytotoxicity of secondary metabolites tested in the present study on the selected cancer cell lines. They observed that cytotoxicity of atranorin on the human cancer cell lines has been much higher when compared to cytotoxicity of gyrophoric acid and parietin. Our results for allelopathic effect of these compounds on lichen algal partner are in accordance with results of Bačkorová et al. (6). Atranorin and gyrophoric acid at highest tested dose (200 μM) were found to be activators of programmed cell death in A2780 and HT-29 cancer cell lines, probably through the mitochondrial pathway of apoptosis (7).

Content of the soluble proteins, when calculated as unit dry weight, was not altered by the presence of all tested secondary metabolites at both selected concentrations (Table 5; Fig. 3A). Levels of hydrogen peroxide and superoxide significantly increased in photobiont cultures due to highest tested concentration of atranorin (Table 5, +72.6% and 260% when compared with respective control cultures; Figs. 3B, 3C). Gyrophoric acid and parietin had no significant effects on production of ROS in photobiont cultures (Table 5). Han et al. (11) found that usnic acid disrupted electron transport in mitochondria and induced oxidative stress in the hepatocytes. Usnic acid increased levels of hydrogen peroxide and superoxide in the cells of *Trebouxia erici* (5). Production of reactive oxygen species in lichens and their symbionts is still an under-researched area of experimental lichenology and requires further study.

Table 5. Content of soluble proteins (mg/g dw), content of hydrogen peroxide ($\mu\text{mol/g dw}$) and content of superoxide ($\mu\text{g/g dw}$) of 2 weeks old *Trebouxia* cultures cultivated on disks with addition of atranorin, gyrophoric acid and parietin (0, 0.01 and 0.1 mg).

Metabolite	Concentration (mg/disk)	Proteins (mg/g DW)	Hydrogen peroxide ($\mu\text{mol/g DW}$)	Superoxide ($\mu\text{g/g DW}$)
	0	58.3 \pm 6.6a	7.29 \pm 1.20b	3.28 \pm 0.47b
Atranorin	0.01	79.8 \pm 19.1a	8.70 \pm 0.78b	5.18 \pm 1.29b
	0.1	87.5 \pm 22.5a	12.58 \pm 2.82a	11.81 \pm 3.06a
Gyrophoric a.	0.01	63.9 \pm 12.1a	7.01 \pm 0.41b	3.24 \pm 0.77b
	0.1	62.2 \pm 9.5a	6.52 \pm 1.03b	3.94 \pm 1.34b
Parietin	0.01	76.8 \pm 13.2a	5.80 \pm 1.41b	4.19 \pm 0.40b
	0.1	63.2 \pm 9.4a	6.66 \pm 2.79b	4.03 \pm 0.94b
	F value	F = 0.94	F = 6.05	F = 14.37
	P value	P = 0.51	P = 0.004	P < 0.001

Values in vertical lines followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

Thus far there is a paucity of information on the toxicity of lichen compounds with the exception of the cytotoxic tests on the effects of usnic acid (dibenzofuran derivative). Usnic acid possesses antimetabolic effects, and this attribute was demonstrated within taxonomically diverse organisms (9). The effect of usnic acid on the spindle apparatus during mitosis was previously confirmed (2). It appears that the action of usnic acid is similar to action of atranorin, as is confirmed in the present study. In our previous study (5) we demonstrated the antimetabolic effect of usnic acid on cultures of aposymbiotically grown photobiont *Trebouxia erici* and free-living alga *Scenedesmus quadricauda*.

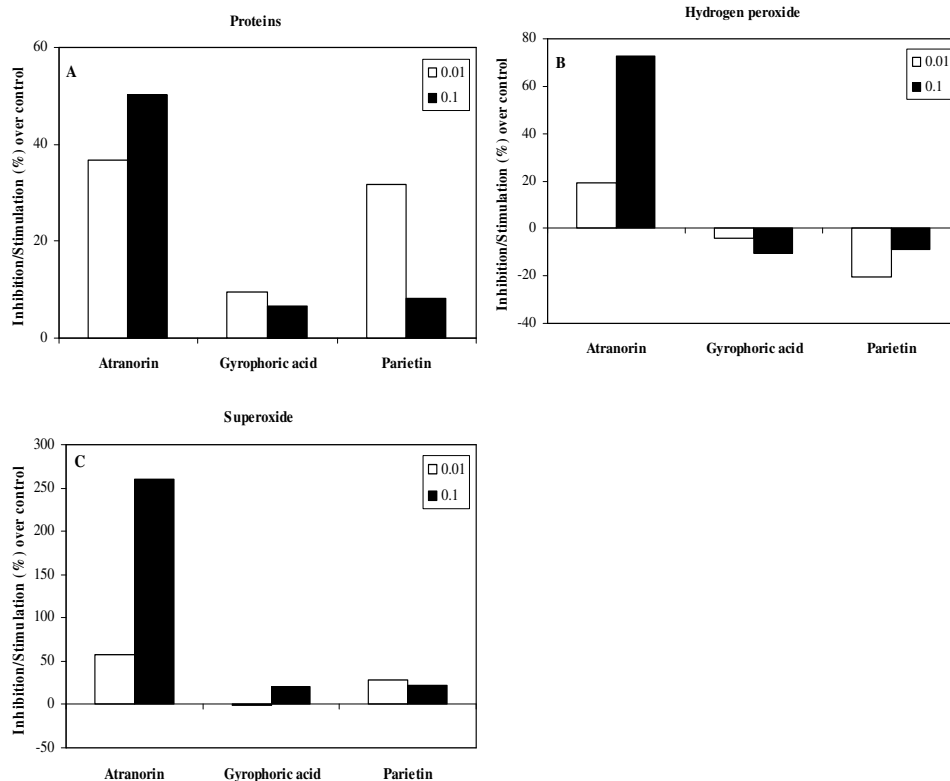


Figure 3. Effects of lichen secondary metabolites on selected physiological parameters of 2 weeks old *Trebouxia* cultures (A: Soluble proteins content, B: Hydrogen peroxide content, C: Superoxide content).

CONCLUSIONS

We conclude that atranorin had phytotoxic effects on lichen photobionts. It seemed that dideside atranorin, cortical lichen metabolite which is in the direct contact with photobiont cell forming layer in lichens with stratified thallus, may act as an allelochemical, controlling the cell division of algal partner inside the thallus thereby regulating the balance between the symbionts. The phytotoxicity of lichen metabolites differed as we did not confirm the phytotoxicity of trideside gyrophoric acid and anthraquinone parietin on photobiont cells at tested concentrations.

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