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Effect of several phenolic compounds of lichens on the physiological, cytological, and biochemical characteristics of *Allium fistulosum* seedlings

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ABSTRACT

Here we report the effects of atranorin (ATR), gyrophoric acid (GPA), and thamnolic acid (TMA) on the physiological, biochemical, and cytological characteristics of *Allium fistulosum* L. seedlings for the first time. Of the lichen substances investigated, ATR and TAM had the highest phytotoxicity effects. ATR (4000 - 1000 μM) and TAM (4000 μM) inhibited the growth processes and increased the intensity of lipid peroxidation in *A. fistulosum*, and as a result the activities of the protective enzymes peroxidase, catalase, and superoxide dismutase increased, and the contents of photosynthetic pigments in the *A. fistulosum* seedlings decreased. ATR at concentrations 2000 and 4000 μM caused decrease of mitotic activity and increase of chromosomal aberrations frequency in root cells. The weakest phytotoxic properties were shown by GPA, which caused no changes in the physiological, cytological, or biochemical parameters of the *A. fistulosum* seedlings across the entire range of concentrations tested.

1. Introduction

Lichens synthesize a number of specific compounds of various chemical types, collectively called 'lichen substances'. Currently, lichen substances form a wide group of metabolites, including 854 compounds (Elix, 2014). The most common types of lichen substances are depsides, which are di- and trimeric compounds; the monomers of these are monocyclic phenolic acids, linked by an ester bond. Many depsides have a wide spectrum of activities, including antibacterial, cytotoxic, and antioxidant activities (White et al., 2014).

Some secondary metabolites of lichens appear to have allelopathic effects on vascular plants and algae (Lechowski et al., 2006; Lokajová et al., 2014). However, little information on the phytotoxic activities of depsides and tripsides is available. Huneck and Schreiber (1972) showed that the potassium salts of atranorin (ATR) and evernic acid, at concentrations of 1 mM, completely inhibited the root growth of cress seedlings.

In previous studies, we investigated the antioxidant activities of some lichen substances (Prokopiev and Filippova, 2019; Sleptsov et al., 2019). We showed that of the eight lichen depsides studied, gyrophoric acid (GPA), thamnolic (TMA) acid, and ATR (Fig. 1) had higher antioxidant activities than both the reference antioxidant dihydroquercetin and other lichen substances.

The purpose of this study was to examine the effects of GPA, TMA, and ATR on the physiological, cytological, and biochemical characteristics of *Allium fistulosum* seedlings.

2. Materials and methods

2.1. Isolation of lichens phenolic compounds

Atranorin (ATR), gyrophoric acid (GPA), and thamnolic acid (TMA) were isolated from lichens *Cladonia rangiferina*, *Umbilicaria pensylvanica*, and *Thamnolia vermicularis*, respectively. To prepare the extracts, 100 g of air-dried lichen thalli were extracted with acetone in a Soxhlet apparatus for 24 h. The extracts were then evaporated to a volume of 20–25 mL on a rotary evaporator. Preparative isolation of the individual substances was performed with the Reveleris® Flash Chromatography System (Grace Davison Discovery Sciences, USA) with a 111 × 10 mm^2 column with C18 silica gel (particle size 40 μm, 12 g) using 0.1% glacial acetic acid solution in water (eluent A) and ethanol (eluent B) as eluents. Elution was performed with an increasing eluent B concentration (10%–100%) at a flow rate of 15 mL/min for 30 min. The volume of extract injected was 3 mL. A fraction collector synchronized with the UV detector was adapted for automatic selection when the absorbance of the solution passing through the measuring cell increased by more than 0.2

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absorbance units (at a wavelength of 210 nm).

The isolated substances were identified by spectral analysis. The infrared spectra were obtained with a Varian 7000 FT-IR Fourier transform infrared spectrometer (Varian Medical Systems, USA) using potassium bromide pellets (400–4000 cm^{-1}). The UV spectra of the substances dissolved in methanol were recorded with a Shimadzu UV-2600 spectrophotometer (Japan) at 190–350 nm. The molecular weights were determined with an Agilent 6538 UHD quadrupole time-of-flight mass spectrometer (Agilent Technologies, USA) with dual electrospray ionization (ESI). The capillary voltage at positive or negative ESI was 3.5 kV; nebulizer gas pressure, 30 psi; dryer gas (nitrogen) temperature, 350°C; dryer gas flow rate, 7 L/min. Ions were recorded within a mass range of 100–1000 m/z. The isolated substances were identified by comparing their molecular weights and IR and UV spectra with those of known lichen metabolites (Huneck, and Yoshimura, 1996).

2.2. Bioassay

The phytotoxicity of ATR, GPA, and TMA at concentrations of 125–4000 μM on seedlings of *A. fistulosum* was studied. The choice of concentrations was based on data obtained in a previous study (Huneck and Schreiber, 1972) which showed that within this concentration range of potassium salts of ATR and some other depsides, there was a significant reduction in laboratory germination and growth inhibition of watercress seedlings. To produce their water-soluble salts, ATR, GPA, and TMA were mixed individually with an aqueous solution of potassium hydroxide. Excess alkalinity was neutralized to pH 7.5–8.0 with HCl.

2.2.1. Physiological studies

The solutions of ATR, GPA, and TMA potassium salts were added to Petri dishes before the seeds were sown. The control group was germinated on distilled water. The seeds were germinated on filter paper (50 pieces in each dish, with four replicates) under a 16 h light period at a temperature of 25 °C in climatic chambers KBWF 720 (Binder, Germany). The rate of germination and the length of the seedling shoots and roots were determined on the 12th day of observation.

2.2.2. Cytological studies

The roots of the seedlings were examined cytologically after they were fixed in Clark's solution for 12 h. The samples were stained with 2% aceto-orcein. The pressed preparations were examined under an Axiostar plus light microscope (Carl Zeiss, Germany). Chromosomal aberrations (CA) were detected with an anaphase–telophase assay (Nefic et al., 2013). The mitotic index (MI), calculated as the ratio of the number of mitotic cells to the total number of cells, expressed as a percentage, was used as a measure of cell division activity (Sabeen et al., 2019).

2.2.3. Biochemical analyses

All spectrophotometric measurements were made on a Shimadzu UV-2600 instrument (Japan). For this study, we took 0.1–0.3 g samples of crude tissue from 12-day-old seedlings and homogenized them in a ceramic mortar with 0.1 M Na-phosphate buffer (pH 7.4) to extract the enzymes or with 96% ethanol and 1% Triton X-100 to extract the thiobarbituric acid reactive species (TBARS). The homogenates were centrifuged for 10 min at 6000×*g* and the supernatants were used for further studies.

2.2.3.1. Superoxide dismutase activity. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed with the modified photochemical method (Giannopolitis and Ries, 1977). The reaction mixture contained 0.1 mL of supernatant and 0.2 mL of 122 mM methionine, 0.2 mL of 0.33 mM riboflavin, 0.1 mL of 1.7 mM nitro blue tetrazolium (NBT), and 2.4 mL of 0.1 M Na-phosphate buffer (pH 7.4). The reaction was initiated by illumination for 5 min with a fluorescent lamp. The enzyme activity was measured as the inhibition of the light-dependent formation of a coloured product during the reduction of NBT ($\varepsilon = 3.98\ mM^{-1}cm^{-1}$, $\lambda = 560$ nm).

2.2.3.2. Peroxidase activity. Peroxidase (POX; EC 1.11.1.7) activity was assayed with the modified method (MacLellan and Robinson, 1981). The reaction mixture contained 0.1 mL of 4.3 mM o-dianisidine solution, 0.7 mL of 0.1 M Na-phosphate buffer (pH 7.4), and 0.1 mL of the supernatant. The reaction was initiated by the addition of 0.1 mL of 0.45 mM hydrogen peroxide. The enzyme activity was determined from the increasing optical density during the formation of the coloured oxidation product of o-dianisidine for 1 min ($\varepsilon = 30\ mM^{-1}cm^{-1}$, $\lambda = 460$ nm) (Vygodina and Konstantinov, 2007).

2.2.3.3. Catalase activity. Catalase (CAT; EC 1.11.1.6) activity was assayed with the method (Aebi, 1984). The reaction mixture contained 0.8 mL of 0.1 M Na-phosphate buffer (pH 7.4) and 0.1 mL of supernatant. The reaction was initiated by the addition of 0.1 mL of 0.45 mM hydrogen peroxide. The enzyme activity was determined from the reduction in optical density during the decomposition of hydrogen

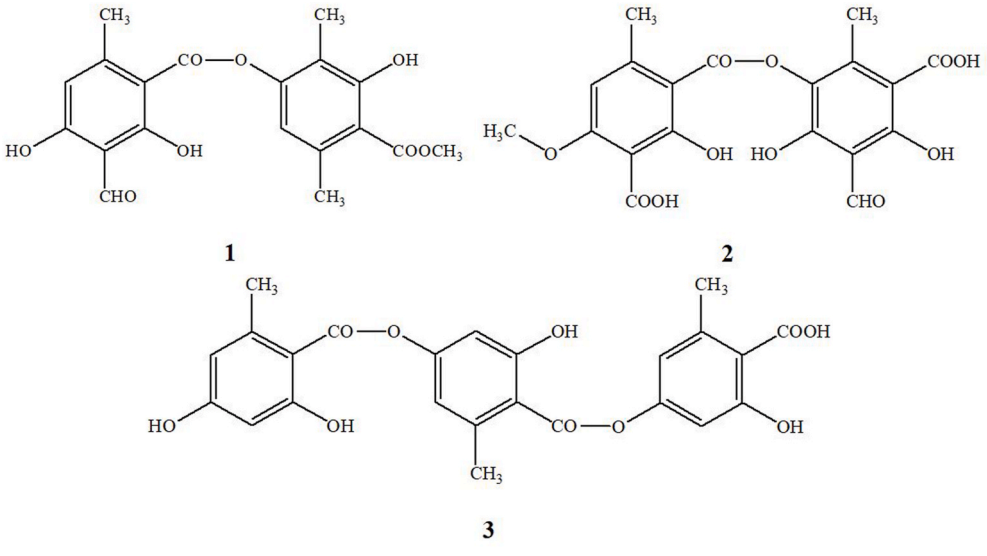

Fig. 1. Chemical structure of atranorin (1), thamnolic acid (2), Gyrophoric acid (3).

peroxide for 1 min (ε = 39.4 $mM^{-1}cm^{-1}$, λ = 240 nm).

2.2.3.4. Lipid peroxidation. The TBARS content was measured according to the modified method (Heath, and Packer, 1968). To determine the TBARS content, 0.5 mL of supernatant was mixed with an equal amount of 20% trichloroacetic acid. The resulting precipitate was separated by centrifugation. One mL of 0.8% thiobarbituric acid was added to the supernatant, and the mixture was incubated at 95 °C for 15 min. The absorbance of the supernatant was measured at 532 nm (ε = 155 $mM^{-1}cm^{-1}$).

2.2.3.5. The photosynthetic pigments content. The concentrations of chlorophylls (a+b) and carotenoids in the shoots (per gram of fresh weight) were determined in 80% acetone extracts at absorbances 663, 647, and 470 nm, respectively (Lichtenthaler, 1987).

2.3. Statistical analysis

The results are presented as arithmetic means and standard deviations (M ± SD) in Tables 1 and 2. The mean values of the samples were compared with one-way analysis of variance (ANOVA). The significance of the differences between the mean values was determined with Dunnett's criterion for multiple comparisons at $P \leq 0.05$. Calculations were performed with the StatPlus v. 2007 statistical analysis program (AnalystSoft, USA).

3. Results and discussion

3.1. Isolation and identification of lichen substances

The following fractions were collected from the acetone extracts of the lichens: *C. rangiferina*, retention time (Rt) 26 min (yield 250 mg) - ATR; *U. pensylvanica*, Rt 12.0 min (300 mg) - GPA; *T. vermicularis*, Rt 15 min (23 mg) - TMA. After removal of the eluent, the dry residue from each fraction was purified by sequential recrystallization from benzene and acetone solutions.

The isolated substances were identified by spectral analysis. The spectra of each substance correspond to those listed in the literature (Table 1), what indicates a sufficiently high degree of the purification process.

Table 1
Results of UV, IR, and ESI-MS spectra of the isolated lichen substances.

Lichen substance	$[M-H]^-$, *m/z*	UV (MeOH), nm	IR(KBr), cm^{-1}
Atranorin ($C_{19}H_{18}O_8$)	373.0929	209, 251, 312[a] 210, 252, 312[b]	783, 802, 821, 860, 937, 990, 1007, 1028, 1076, 1106, 1163, 1200, 1214, 1236, 1268, 1284, 1352, 1378, 1408, 1451, 1583, 1651[a] 790, 810, 830, 870, 960, 990, 1010, 1030, 1078, 1108, 1166, 1198, 1210, 1250, 1270, 1285, 1360, 1382, 1410, 1458, 1580, 1652, 3000, 3500[b]
Gyrophoric acid ($C_{24}H_{20}O_{10}$)	467.0982	216, 270, 307[a] 214, 271, 305[b]	695, 737,794, 831, 861, 892, 973, 993, 1048, 1070, 1144,1201, 1242, 1312, 1349, 1381, 1444, 1463, 1503, 1608, 1640, 1657, 3049, 3094, 3527[a] 700, 740, 800, 840, 870, 900, 985, 1000, 1050, 1070, 1140, 1200, 1240, 1310, 1350, 1385,1450, 1465, 1508, 1610, 1640, 1665, 3050, 3150, 3450[b]
Thamnolic acid ($C_{19}H_{16}O_{11}$)	419.0615	216, 260, 312[a] 218, 260, 313[b]	660, 682, 720, 761, 822, 868, 958, 970,1012, 1043, 1057, 1100, 1121, 1175, 1190, 1232, 1250, 1382, 1415, 1450, 1584, 1630, 1740, 2608, 2705, 2995, 3294[a] 665, 684, 720, 762, 820, 870, 958, 970,1010, 1045, 1058, 1100, 1120, 1175, 1195, 1230, 1250, 1380, 1415, 1455, 1580, 1630, 1740, 2600, 2700, 3000, 3300[b]

[a] Experimental data.
[b] Data from reference (Huneck, and Yoshimura, 1996).

3.2. Physiological studies

The phytotoxicity of the lichen substances on seedlings of *A. fistulosum* was tested at concentrations of 125–4000 μM. Concentrations of 1000–4000 μM ATR caused statistically significant reduction (1.3–2.5 ×) germination as well as (1.2–2.1 ×) shoot and (1.2–3.5 ×) root lengths relative to the control (Table 2). Concentrations of 4000 μM TMA caused reduction (1.3 ×) shoot and root lengths relative to the control. GPA exert no significant effect on the physiological characteristics of *A. fistulosum* seedlings in the concentration range studied. The inhibition of growth processes was observed during treatment with 1000 μM ATR, which is consistent with previously reported data (Huneck and Schreiber, 1972).

3.3. Cytological studies

It is known that some phenolic lichen compounds are genotoxic effects and may slow down the division of meristem cells (Leandro et al., 2013; Öztürk et al., 1999). In fact, mitotic index (MI) and chromosomal aberrations (CA) assays, as short-term cell proliferation and genotoxicity assays, are widely used to monitor chemically-induced damage (Nefic et al., 2013). Previously, study of usnic acid enantiomers (widespread lichen dibenzofuran) genotoxicity in *A. fistulosum* seedlings showed that mutagenic effect revealed by the DNA comet method correlated well with the data on the CA frequency over the entire concentration range studied (Prokopiev and Filippova, 2020).

ATR at concentration 2000 and 4000 μM caused a statistically significant decrease (1.3 and 2.2 ×) of MI and increase (1.2 and 1.5 ×) of CA frequency, which indicates on cytostatic and genotoxic effects of this compound. No statistically significant changes in the MI or CA were detected after treatment with GPA or TMA, which indicates that these substances exert no mutagenic effect in the concentration range studied.

3.4. Biochemical studies

3.4.1. Effect of ATR, TMA and GPA on the antioxidant characteristics of seedlings

Concentrations of 1000–4000 μM ATR and 4000 μM TMA caused a statistically significant increase 1.7–2.4 × and 1.6 × respectively in the concentration of thiobarbituric acid reactive species (TBARS) in the cells of the *A. fistulosum* seedlings (Table 3). The overproduction of TBARS indicates the activation of lipid peroxidation processes in seedling cells. Although on the one hand, ATR is an antioxidant, on the other hand, in concentrations above 300 μM ATR presented a pro-oxidant capacity in a lipid-rich system, enhancing TBARS formation induced (Melo et al., 2011). A similar effect is characteristic of another well-studied lichen substance, usnic acid, which also began to exhibit pro-oxidant properties in lymphocyte cells at concentrations above 100 μM (Kohlhardt-Floehr et al., 2010). One possible explanation might be that ATR and TMA can act as a protonophore uncoupler caused an uncoupling of oxidative phosphorylation in mitochondria and inhibited mitochondrial respiration which might cause hyperproduction of reactive oxygen species (ROS) in cells (Abo-Khatwa et al., 1996; Demine et al., 2019).

Hyperproduction of ROS and activation of lipid peroxidation in cells can initiate the mobilization of responses that can significantly increase the antioxidant potential of plants (Kreslavski et al., 2012).

Table 2
Physiological and cytological parameters of *Allium fistulosum* seedlings under the influence of various concentrations of atranorin, gyrophoric acid, and thamnolic acid.

Concentration, μM	Germination, %	Shoot length, mm	Root length, mm	Mitotic index, %	Chromosomal aberrations, %
0	80.0 ± 4.0	39.6 ± 1.1	29.6 ± 1.2	10.3 ± 0.9	14.1 ± 1.6
Gyrophoric acid					
125	76.3 ± 5.0	41.8 ± 0.9	32.4 ± 1.5	12.1 ± 0.7	16.2 ± 1.1
250	80.7 ± 5.0	41.1 ± 1.1	32.0 ± 1.4	12.5 ± 0.5	14.6 ± 2.1
500	80.0 ± 8.7	41.5 ± 1.2	31.0 ± 1.3	12.4 ± 0.4	17.5 ± 3.0
1000	74.7 ± 4.2	39.6 ± 1.2	29.2 ± 1.3	11.5 ± 0.5	18.0 ± 2.7
2000	81.6 ± 4.0	35.1 ± 0.6	27.8 ± 0.8	9.7 ± 0.3	15.0 ± 2.2
4000	74.0 ± 1.5	35.3 ± 0.8	26.4 ± 1.4	9.3 ± 0.5	14.6 ± 2.0
Atranorin					
125	82.7 ± 4.2	38.8 ± 1.0	28.9 ± 1.5	9.7 ± 0.4	13.0 ± 1.9
250	76.0 ± 8.7	38.1 ± 0.8	27.8 ± 1.5	12.0 ± 0.5	13.6 ± 2.9
500	80.7 ± 4.2	37.8 ± 1.0	26.6 ± 1.5	11.7 ± 0.4	10.3 ± 1.6
1000	64.3 ± 4.7*	33.6 ± 0.9*	24.9 ± 1.4*	12.5 ± 0.8	17.0 ± 1.2
2000	53.4 ± 3.0*	24.2 ± 2.0*	11.8 ± 1.8*	7.8 ± 0.9*	27.4 ± 2.4*
4000	32.6 ± 3.6*	19.0 ± 2.5*	8.3 ± 1.9*	4.6 ± 0.6*	31.1 ± 5.8*
Thamnolic acid					
125	74.7 ± 1.2	38.2 ± 0.8	29.6 ± 1.3	11.2 ± 0.6	16.9 ± 2.7
250	76.7 ± 4.2	39.4 ± 1.4	28.0 ± 1.6	11.5 ± 0.3	13.4 ± 1.4
500	73.0 ± 4.0	39.0 ± 1.1	28.1 ± 1.5	11.9 ± 0.5	15.7 ± 2.4
1000	72.7 ± 4.2	36.9 ± 1.0	26.4 ± 1.5	12.3 ± 1.0	16.4 ± 1.6
2000	71.4 ± 1.5	37.9 ± 2.5	28.2 ± 0.7	10.0 ± 0.6	13.9 ± 1.7
4000	77.0 ± 2.1	31.0 ± 2.0*	22.2 ± 2.6*	10.7 ± 0.8	15.3 ± 2.2

Data are represented as mean ± standard deviation. *- Differences are statistically significant compared with the control ($p < 0.05$, ANOVA, Dunnett's test). 50 seeds with four replicates were used to determine the physiological parameters of seedlings. 20 roots of the seedlings from each concentration were used for the calculation of mitotic index and chromosomal aberrations frequency.

Table 3
Biochemical characteristics of *Allium fistulosum* seedlings under the action of various concentrations of atranorin, gyrophoric acid, and thamnolic acid.

Concentration, μM	TBARS, nM/g FW	CAT, nM/min g FW	POX, mM/min g FW	SOD, mM/min g FW	Chlorophylls μg/g FW	Carotenoids, μg/g FW
0	15 ± 3	212 ± 19	2.8 ± 0.2	1.8 ± 0.2	342 ± 23	90 ± 10
Gyrophoric acid						
125	14 ± 2	207 ± 20	2.6 ± 0.2	1.9 ± 0.2	333 ± 12	84 ± 5
250	16 ± 2	189 ± 18	2.7 ± 0.3	2.2 ± 0.2	367 ± 22	95 ± 15
500	14 ± 3	202 ± 13	2.7 ± 0.3	2.2 ± 0.2	335 ± 10	80 ± 9
1000	15 ± 2	199 ± 17	2.5 ± 0.2	2.3 ± 0.2	351 ± 16	91 ± 12
2000	16 ± 1	210 ± 19	2.6 ± 0.2	2.0 ± 0.2	349 ± 20	79 ± 9
4000	17 ± 2	214 ± 17	2.8 ± 0.3	2.2 ± 0.3	348 ± 18	80 ± 9
Atranorin						
125	19 ± 2	209 ± 26	2.6 ± 0.2	1.8 ± 0.2	350 ± 28	92 ± 14
250	20 ± 3	224 ± 22	2.8 ± 0.2	1.9 ± 0.2	322 ± 13	83 ± 6
500	20 ± 3	258 ± 24*	3.1 ± 0.2	2.4 ± 0.2*	311 ± 10	81 ± 5
1000	25 ± 2*	288 ± 18*	3.4 ± 0.2*	2.6 ± 0.2*	284 ± 22*	66 ± 13*
2000	32 ± 4*	316 ± 21*	4.9 ± 0.7*	3.1 ± 0.3*	275 ± 22*	64 ± 6*
4000	35 ± 2*	259 ± 26*	3.3 ± 0.2*	2.7 ± 0.3*	260 ± 24*	60 ± 5*
Thamnolic acid						
125	16 ± 2	225 ± 13	2.7 ± 0.3	1.8 ± 0.2	373 ± 28	110 ± 10
250	17 ± 2	218 ± 18	2.6 ± 0.2	1.8 ± 0.2	380 ± 21	104 ± 14
500	18 ± 2	207 ± 23	2.7 ± 0.3	2.1 ± 0.1	372 ± 23	105 ± 10
1000	19 ± 3	215 ± 14	2.5 ± 0.2	2.0 ± 0.2	384 ± 20	90 ± 7
2000	21 ± 3	238 ± 11	3.3 ± 0.2*	2.5 ± 0.2*	351 ± 21	84 ± 8
4000	23 ± 2*	261 ± 11*	3.6 ± 0.3*	2.7 ± 0.2*	294 ± 19*	63 ± 7*

Data are represented as mean ± standard deviation. *- Differences are statistically significant compared with the control ($p < 0.05$, ANOVA, Dunnett's test). TBARS - thiobarbituric acid reactive species; CAT – catalase; POX – peroxidise; SOD – superoxide dismutase.

Consequently, 500–4000 μM ATR and TMA 2000–4000 μM increased the activities of antioxidant enzymes, such as CAT (1.2–1.4 and 1.1 ×), POX (1.2–1.8 and 1.2–1.3 ×), and SOD (1.3–1.7 and 1.3–1.4 ×), respectively compared with the control. GPA exert no significantly effect on antioxidant enzymes activities in the concentration range studied.

It can be assumed that ROS overproduction in the concentration range of ATR (125–500 μM) and TMA (125–2000 μM) was compensated by the activation of protective antioxidant systems, which ensure a balanced occurrence of redoxreactions in the tissues of *A. fistulosum* seedlings. At the same time under the action of higher concentrations, a shift in the prooxidant-antioxidant balance towards the activation of lipid peroxidation and the development of oxidative stress was observed.

Previously, studied the effect of usnic acid enantiomers on *A. fistulosum* seedlings. (+) and (−) usnic acid exhibit phytotoxic effects due to the development of oxidative stress at concentrations above 62.5 μM was shown (Prokopiev and Filippova, 2020). At the same time, the studied depsides caused the formation of oxidative stress only at 1000 (ATR) and 4000 (TMA) μM which indicates their significantly lower toxicity on plants.

3.4.2. *Effect of ATR, TMA and GPA on the photosynthetic pigments*

A dose-dependent depigmentation of *Lactuca sativa* and *A. cepa* seedlings treated with some lichen metabolite previously reported (Romagni et al., 2000). ATR (4000–1000 μM) and TMA (4000 μM) also reduced the contents of chlorophylls 1.2–1.3 × and carotenoids 1.4–1.5

× in the *A. fistulosum* seedlings, respectively (Table 3). Previous work has demonstrated that at an ATR concentration of 0.23 mg/mL, the permeability of oak chloroplast membranes increased and the chlorophylls were degraded (Bouaid and Vicente, 1998). Those authors suggested that the degradation of chlorophylls was attributable to the loss of Mg^{2+} ions from various molecules after chelation by ATR.

4. Conclusion

ATR exhibits the highest toxicity among the three studied lichen depsids on seedlings of *A. fistulosum*. The phytotoxic effects of ATR and TMA are associated with oxidative stress. Pro-oxidant properties of ATR can be useful in the development of new anticancer drugs and/or herbicides. The weakest toxicity of those three depsids is GPA, which at the same time has a high antioxidant activity. It opens the possibility for its further use in the cosmetic and food industries. The results indicate the promise of further studies of lichens as a source of biologically active compounds for use in the pharmaceutical and agricultural industries in the future.

CRediT authorship contribution statement

Ilya Prokopiev: Supervision, Investigation, Data curation. Igor Sleptsov: Conceptualization, Writing - original draft, Formal analysis. Galina Filippova: Methodology, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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