https://doi.org/10.26651/allelo.j/2019-47-2-1233 0971-4693/94 Euro 20.00
Allelopathy Journal 47 (2): 221-232 (July, 2019) *International Allelopathy Foundation* 2019
Table: 1, Figs : 3

Host species affects the phenolic compounds content in *Hypogymnia physodes* (L.) Nyl. thalli

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(Received in revised form: March 27, 2019)

ABSTRACT

We studied the differences in the concentration of secondary metabolites in *Hypogymnia physodes* thalli, growing on various woody species and the relationship between the content of these compounds and chemical properties of the host bark, i.e., pH and concentration of plant phenolics. The quantitative variations in the accumulation of physodalic, 3-hydroxyphysodic, physodic acids and atranorin in thalli from deciduous (willow, ash, birch and hazel) and coniferous (spruce) species were determined by HPLC method. The total lichen substance content varied from 11.5% to 14.4% of thallus dry weight depending on the host species. The accumulation of physodic acid, 3-hydroxyphysodic acid, physodalic acid and atranorin in the thalli ranged 30-57%, 22-29%, 7-24% and 6-24% of the total secondary metabolites content, respectively. Physodalic acid and atranorin levels were highest in thalli growing on willow, but 3-hydroxyphysodic acid and physodic acid contents were highest in thalli on spruce and ash bark, respectively. Bark phenolics had significant effects on physodalic and physodic acids content, while bark pH - on 3-hydroxyphysodic acid content. Our results suggested that species-specific bark characteristics affects the production of secondary metabolites in epiphytic lichens. These findings indicate significant role of substratum in regulation of secondary compounds production in lichens.

Key words: Ash, bark pH, *Betula pendula*, birch, *Corylus avellana*, *Fraxinus exelsior*, hazel, HPLC, *Hypogymnia physodes*, lichen compounds, *Picea abies*, plant phenolics, *Salix alba*, secondary compounds, spruce, willow, woody host species.

INTRODUCTION

Lichen-forming fungi grow in soils, on rocks, trees, shrubs, other organisms and various anthropogenic objects (35). Many epiphytes show a narrow ecological tolerance and are associated with specific substratum qualities. Thus, they are limited to one or few host tree species, for example, the corticolous *Lecanora populicola*, which is specialist of aspen (*Populus tremula* L.) (38). Others, such as *Hypogymnia physodes*, are ubiquitous and live on various coniferous and deciduous trees bark, without showing strict substratum preferences and on wood, rocks and other materials (38). The significance of substrate factors (*i.e.* trees species, trunk diameter, roughness, concentration of phenols and nutrients in bark, pH) for epiphytic lichen growth had been investigated (8,15,36). Some studies indicated that the tree species itself is major factor affecting the lichen colonization (1,40).

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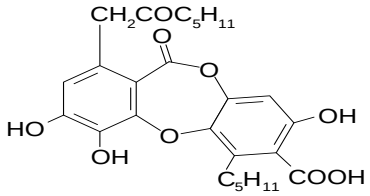
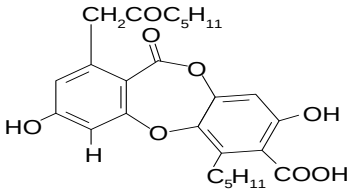
As long-living and slow-growing organisms, lichens produce many chemicals that protect them against abiotic environmental stress factors: [Excessive UV and photosynthetically active radiation (PAR) (7,39)], desiccation (21), limited or high ion content (20) and substratum acidity (17). Thus, secondary metabolites allow lichens to adapt to different environmental conditions (26). These chemicals can also exert allelopathic effects on lichens' pathogens, competitors and consumers (9,26,27). In our previous study (25), we suggested that lichen substances produced by *H. physodes*, covering large part of spruce bark, were responsible for decreasing the host trees vigour. On the other hand, Koopmann *et al.* (23) demonstrated negative allelopathic effects of plant phenolic compounds derived from the hydrolytic decomposition of trees bark on the growth of lichen's soredia.

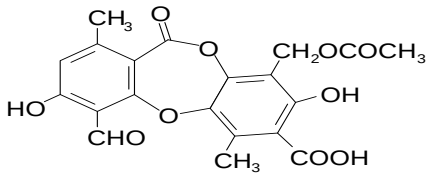
The total content of lichen compounds and their relative proportions in the thalli are based on their biosynthesis and degradation rates (11). These processes are influenced by many environmental parameters : [Solar radiation **(**light and UV), temperature, moisture, seasonal variation, geographical latitude and altitude, metal ion concentration in substrate and air pollution (4,5,6,14,16,32,39)]. The culture conditions, including the type of medium, availability of nutrients and pH influences the amount and the profile of compounds produced by aposymbiotically cultivated lichen mycobionts (37,44). Therefore, it can be assumed that the properties of substratum can affect the production of secondary metabolites by lichens growing in natural conditions. In epiphytes, the chemical and physical properties of bark of woody host species are important. The impact of environmental factors on the content of lichen compounds in thalli is well known (10,37), but the influence of substrate characteristic is little. Recent studies have shown that the content of lichen phenolics in *H. physodes* growing on spruce bark was correlated with heavy metal concentrations in the substratum (16).



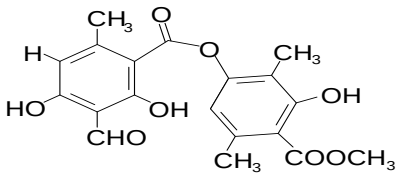
3-Hydroxyphysodic acid
(orcinol depsidone)



Physodic acid
(orcinol depsidone)



Physodalic acid
(*β*-orcinol depsidone)



Atranorin
(*β*-orcinol *para*-depside)

Figure 1. Chemical structures of the major secondary metabolites produced by *H. physodes*.

H. physodes produces at least ten secondary metabolites [*β*-orcinol depsidones (protocetraric, physodalic and conphysodalic acids), orcinol depsidones (3-hydroxyphysodic, physodic, 2'-*O*-methylphysodic, 4-*O*-methylphysodic, and *α*-alectoronic acids), as well as *β*-orcinol *para*-depsides (atranorin and chloroatranorin) (24)]. Their content varies up to 20% of the thallus dry weight (39). All known compounds produced by *H. physodes* are phenolics biosynthesized by the acetyl-polymalonyl pathway (polyketide pathway) by multidomain enzyme type I polyketide synthases (PKSs) (3,42). However, the formation of orcinol- and *β*-orcinol-type compound precursors is catalysed by two different type I PKSs without or with methyl transferase subunit (28,42,43).

In the present study, we hypothesized that the secondary metabolite content in epiphytic lichen thalli depends on the bark characteristics of host. We analysed differences in *H. physodes*, concentrations of 4-major phenolics, [physodalic, 3-hydroxyphysodic and physodic acids, and atranorin (Fig. 1)], in thalli growing on willow, ash, birch and hazel (deciduous) and spruce (coniferous). As the substance contents in lichen thalli is affected by the environmental factors (37), we chose woody species growing in close proximity and in similar habitat conditions (solar radiation, temperature, and precipitation). Moreover, we estimated the relations between the selected chemical properties of host bark (pH, and total phenolics concentration) and the secondary metabolites content in *H. physodes* thalli.

MATERIALS AND METHODS

Materials

The lichen, *H. physodes* and the 4-deciduous woody species: willow (*Salix alba* L.), ash (*Fraxinus excelsior* L.), birch (*Betula pendula* Roth) and hazel (*Corylus avellana* L.), and one coniferous species, spruce (*Picea abies* (L.) Karst.) were chosen for the analyses. All samples were collected from the natural mixed forest in Ochotnica Górna, South Poland (49°32' N, 20°19' E) on 29 September 2015. The individuals trees and shrubs were sampled randomly from the southern edge of the forest in about 1500 m length and depth of about 20 m. The forest in the collection area had open canopy cover of about 25 m height. Only the hazel shrubs were about 5 m high. The age of sampled trees was 30 to 50 years. Bark fragments containing cork (outer bark), cork cambium and living phloem outside the vascular cambium were sampled from young live branches (2-3 cm dia) of trees and shrubs, 30-40 years old. The bark samples were collected in 10-replicates for each woody species; one piece of bark per specimen. Based on our previous study (25), which showed the allelopathic effects of *H. physodes* thalli on the spruce bark chemistry, including the alteration of protein and phenolics contents and antioxidative enzymes activity (unpublished data), we decided to take samples of bark fragments from trees or shrubs without lichen thalli. Thus, the lichen and bark samples came from different individuals. The lichen was identified using standard keys (30,38) and harvested in ten replicates for each host species (one thallus from each specimen). A voucher specimen was deposited in the Herbarium collection, Department of Plant Physiology and Development, Jagiellonian University, Poland.

Extraction of lichen secondary metabolites

The lyophilized freeze-dried lichen thalli (50 mg dry weight, DW) were powdered in a homogenizer and extracted in triplicate with 1 mL methanol for 30 min at 20 ± 1°C. After each extraction, they were centrifuged at 15,000×*g* for 10 min. The supernatants were pooled and

evaporated to dryness under a stream of nitrogen. The dry residues of extracts were re-dissolved in 1 mL of high-performance liquid chromatography (HPLC) grade methanol. They were stored at -20°C for 1-2 days for HPLC analysis.

HPLC analysis

The HPLC system (Waters Inc., Milford, MA, USA), equipped with a 717 plus autosampler, a 600E gradient pump, a Jetstream 2 plus column thermostat, a 996 photodiode array (PDA) detector and Millenium^{32} SS software with the PDA option was used. The lichen thalli methanol extract was filtered through PTFE membrane filters (0.45 μm) and injected at a volume of 20 μL into an analytical reverse-phase HPLC column (Nova-Pack C18, 4 μm, 4.6×250 mm, Waters, Inc.) maintained at 25°C. The separation was achieved using the method described by Feige *et al.* (12) with some modifications. The gradient mobile phase consisted of water (A) and methanol (B), both acidified with 0.1% (v/v) formic acid was pumped at a flow rate of 1 mL min^{-1} starting with 10% B. Elution was conducted using the following steps: 15 min, 65% B; 60 min, 80% B; 65 min, 100% B; 75 min, 100% B; 85 min, 10% B. Finally, the column was equilibrated for 15 min before the next run. Absorbance was measured from 200 to 450 nm and compared with that in the literature (22). Chromatogram profile was automatically recorded at 254 nm.

Preparation of *H. physodes* secondary metabolites standards

The standards of *H. physodes* secondary metabolites are not commercially available, hence, they were prepared as under: During the separation of extract of thalli growing on spruce bark, the fractions corresponding to the peaks clearly differed from the baseline were isolated and evaporated to dryness. Next, the compounds were qualitatively identified by mass spectrometry (MS). For the MS analysis, the dry residues of post-collection peak fractions were re-dissolved in 0.5 mL of 30% methanol containing 0.1% formic acid and analysed with an Esquire 3000 quadrupole ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany) fitted with an electrospray ion source (ESI-MS). The following operating conditions were applied: heated capillary temperature at 280°C, drying gas (high-purity nitrogen) flow rate of 6 L min^{-1}, capillary voltage of 4.5 kV and cone voltage of 25 V. The samples were measured in the negative-ion mode and scanned in the range of 50-600 *m/z* in both the MS and MS/MS analyses. Then, the molecular ion masses and fragmentation patterns were compared with literature data (24). Compounds serving as standards of *H. physodes* metabolites were gravimetrically purified to prepare the calibration curves. For this purpose, they were collected during several HPLC separations, then lyophilized and weighed on the analytical balance (HM-202-EC, A&D Instruments Ltd., Japan) with a resolution of 0.1 mg. The calibration curves were prepared for each metabolite based on three different compound concentrations in methanol using linear regression analysis. The area of chromatographic peaks was used for quantification.

Identification of lichen compounds

The *H. physodes* secondary metabolites from the various lichen thalli extracts were qualitatively identified by comparing their retention times (R_t) and absorbance spectra with those of prepared standards. The individual lichen metabolite content in thalli were calculated based on calibration curves prepared for the gravimetrically purified standards.

Measurement of chemical bark properties

***pH* :** The lyophilized and powdered bark samples (100 mg DW) were mechanically shaken with 1 mL deionized water at room temperature for 24 h. The pH value of suspension was determined using a Microcomputer pH meter CP-315M (Elmetron, Poland) with an InLab®423 pH Micro Electrode (Mettler-Toledo, Greifensee, Switzerland).

***Total extractable phenolics*:** The bark samples were lyophilized, powdered and extracted in 80% methanol. Total phenolics content in the extracts were determined spectrophotometrically (spectrophotometer V-650, Jasco, Japan) using the Prussian blue assay as per Price and Butler (34) with modifications. A total of 2.5 mL of deionized water and 150 µL of 0.1 M $FeNH_4(SO_4)_2$ in 0.1 M HCl were added to 25 µL of the bark extract, then shaken and left for 20 min. Next, 150 µL of 8 mM $K_3Fe(CN)_6$ were added, and after an additional 20 min, the absorbance was measured at 720 nm. Solvent solution without extract was used as blank. Total extractable phenolics content ($mg \cdot g^{-1}$ DW) was calculated using the standard curve for gallic acid and expressed as its equivalent.

Chemicals

Analytical grade methanol, hydrochloric acid, ammonium iron (III) sulfate dodecahydrate and potassium ferricyanide were purchased from Avantor Performance Materials (Gliwice, Poland). The HPLC grade reagents used were methanol from J.T. Baker (Phillipsburg, NJ, USA) and formic acid and gallic acid from Sigma-Aldrich (St. Louis, MO, USA). Deionized water used in the analyses was generated by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Statistical analysis

After Levene's test to assess the equality of variances, a one-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to verify the differences in bark pH, plant phenolics total accumulation as well as lichen secondary metabolites content across particular woody species. Prior to the analysis, the distribution normality of variables was verified using the Kolmogorov-Smirnov test. The relationships between bark parameters and particular lichen secondary metabolite content were analysed by calculating Pearson correlation coefficients. The analysis was performed on averaged values for particular host species.

RESULTS AND DISCUSSION

Secondary metabolite content in *H. physodes* thalli

HPLC analysis of the *H. physodes* thalli extracts from the different woody tree species samples revealed the presence of same four major lichen compounds, these were identified in elution order as physodalic acid, 3-hydroxyphysodic acid, physodic acid and atranorin (Fig. 2). This finding is in accordance with earlier results that *H. physodes* is qualitatively uniform and has only one chemotype (32).

The total content of lichen compounds in the thalli from different tree woody hosts varied from 11.5% DW in the thalli colonizing ash branches to 14.4% DW in the thalli growing on willow branches. The percentage contribution of individual compounds in the total concentration of *H. physodes* secondary metabolites followed the order : physodic acid > 3-hydroxyphysodic acid > physodalic acid > atranorin. Only the thalli harvested from the

willow bark showed different pattern in metabolites concentration: physodic acid > physodalic acid > atranorin > 3-hydroxyphysodic acid. The accumulated physodic acid, 3-hydroxyphysodic acid, physodalic acid, and atranorin in lichen thalli comprised 30-57%, 22-29%, 7-24% and 6-24% of the total secondary metabolites content, respectively. These results indicated that physodic acid was dominant compound in all tested thalli, irrespective of host species. Its content fluctuated from 4.26% DW in the thalli from willow branches to 6.51% DW in the thalli from ash branches (Fig. 3).

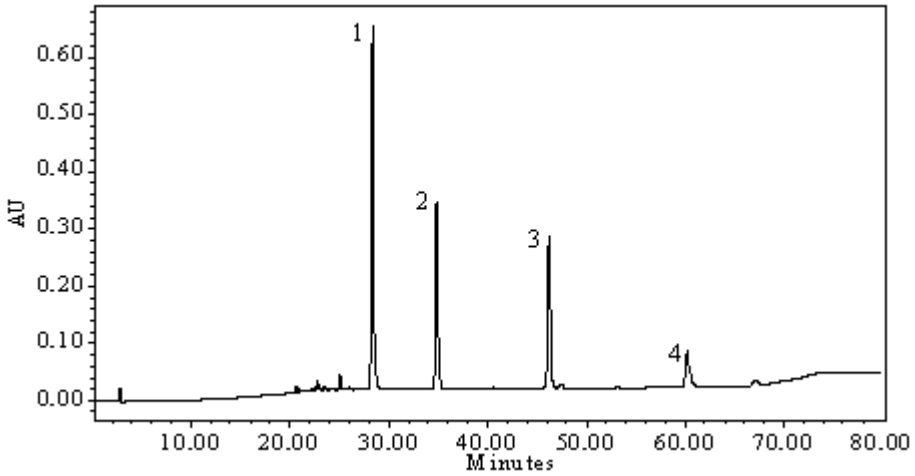


Figure 2. Exemplary HPLC chromatogram of methanol extract of *H. physodes* thalli growing on spruce. Analysed lichen secondary metabolites: 1 : Physodalic acid, 2 : 3-Hydroxyphysodic acid, 3 : Physodic acid, 4 : Atranorin. Monitored at wavelength of 254 nm.

The host species differed from each other with the content of particular lichen compounds in thalli (Fig. 3). Significantly highest concentrations of atranorin and physodalic acid were found in the thalli from the willow branches (about 4-times more than from ash branches) (Fig. 3). Significantly highest contents of 3-hydroxyphysodic acid and physodic acid were found in the thalli that colonized the spruce and ash bark, respectively (Fig. 3). Some lichen compounds were quantitatively related to each other. For example, the accumulation of physodic acid was strongly negatively correlated with the physodalic acid and atranorin content (Table 1). The strong positive correlation between the physodalic acid and atranorin contents (Table 1) could be explained by their common biosynthetic pathway, in which methyl-3-orsellinate, a precursor of *β*-orcinol-type compounds, is formed (28,43). This negative correlation between the accumulation level of these two substances and the concentration of physodic acid, belonging to the orcinol-type group of lichen compounds.

Significant variations were found in the *H. physodes* secondary metabolites content, particularly biosynthetically related physodalic acid and atranorin (Fig. 3), in the thalli growing on various host species. This suggested that activity of the proper type I polyketide synthases and/or the expression of PKSs-coding genes might depend on the species-specific bark parameters. However, detailed investigations on this hypothesis have not been conducted. Some factors e.g., substrate pH, regulates the PKS gene expression in fungi and lichen mycobionts (33,41,44).

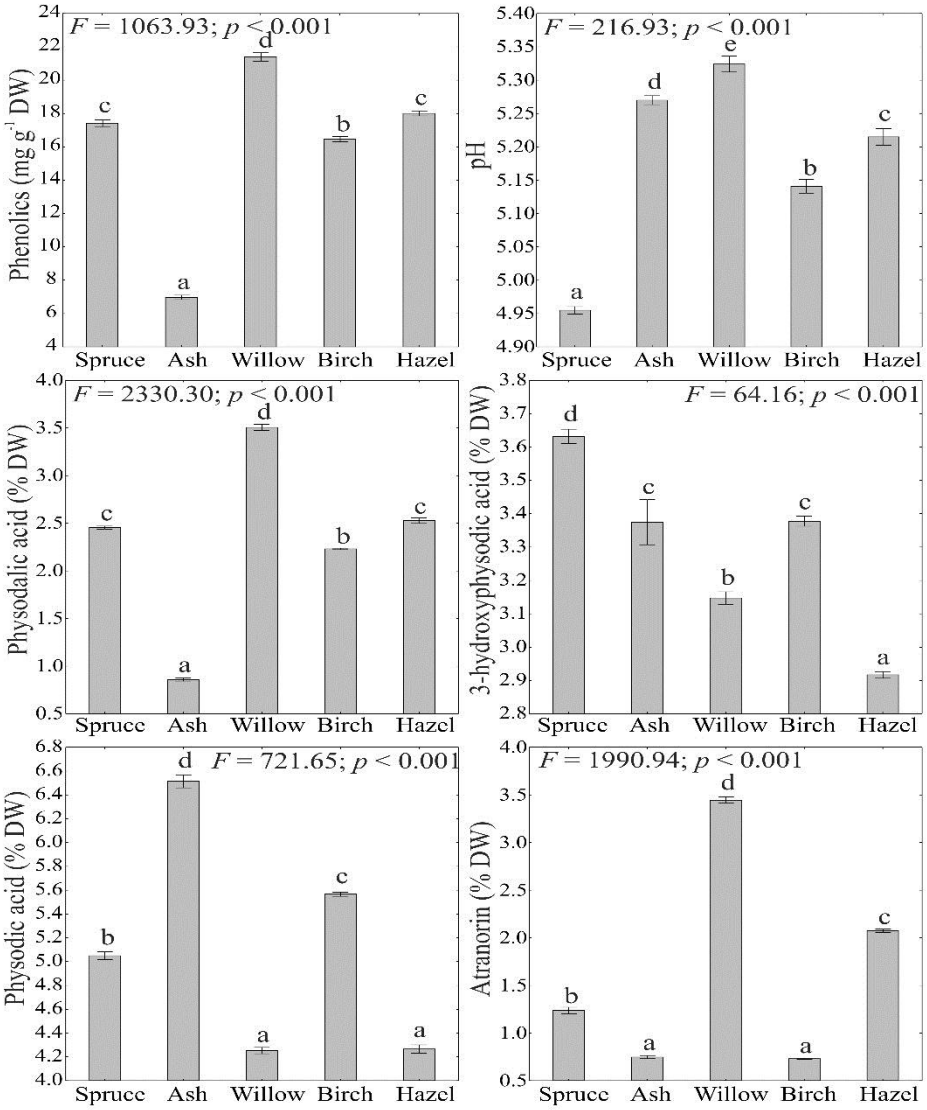


Figure 3. Bark parameters (pH and total concentration of plant phenolics) and secondary metabolites content in lichen thalli for particular woody species. Results of ANOVA (p<0.05); F and p values are provided. Letters denote the results of Tukey's (HSD) test; different letters indicate significant differences at the p<0.05 level. Values are means ± standard error (SE) (n = 10).

Chemical parameters of host bark and the lichen metabolites content in the thalli

Bark from the tested coniferous and deciduous woody species differed in their pH and concentrations of phenolic compounds (Fig. 3). The bark pH of all woody hosts were typical for the relevant tree and shrub species (13,29,40) and changed as follows: willow

(5,32) > ash (5,27) > hazel (5,22) > birch (5,14) > spruce (4,96). Although the bark pH was always acidic, there were significant differences in the pH between the host species. However, this bark parameter had significant effects on the content of only one secondary metabolite produced by *H. physodes* thalli. The content of 3-hydroxyphysodic acid was negatively correlated with bark pH; the more acidic pH gave higher content of this compound (Table 1). The level of other lichen secondary metabolites did not show clear relationship with pH (Table 1). *H. physodes* chosen for our study is characterized by high tolerance to air and substratum pollution, as well as the ability to adapt to varying environmental conditions, including pH, which gives it a ubiquitous character (31,35). As previously suggested (17), some lichen compounds, including usnic, norstictic, fumarprotocetraric, perlatolic and thamnolic acids, play an important role in controlling the acidity tolerance in lichens, due to their physicochemical characteristics (primarily pK_{a1} values). This shapes the ecological preference of lichens for specific pH conditions and determines the range and diversity of lichen species (18,19). Thus, lichen species, depending on the metabolites they produce, are characterized by varying degrees of sensitivity to pH changes in the substratum (17,18). It is possible that 3-hydroxyphysodic acid is involved in mechanism that leads to the high adaptability of this lichen species.

Table 1. Pearson correlation coefficients (*r*) for bark parameters (bark pH, total accumulation of plant phenolics) and lichen secondary metabolites content

	Phenolics	Physodalic acid	3-Hydroxy physodic acid	Physodic acid	Atranorin
pH	*r* = -0.0979	*r* = 0.0364	***r* = -0.6872**	*r* = -0.0565	*r* = 0.4892
	p = 0.876	*p* = 0.954	***p* = 0.048**	*p* = 0.928	*p* = 0.403
Phenolics		***r* = 0.9813**	*r* = -0.3006	***r* = -0.9166**	*r* = 0.7149
		***p* = 0.003**	*p* = 0.623	***p* = 0.029**	*p* = 0.175
Physodalic acid			*r* = -0.3236	***r* = -0.9094**	***r* = 0.8197**
			p = 0.595	***p* = 0.032**	***p* = 0.049**
3-Hydroxyphysodic acid				*r* = 0.5595	*r* = -0.5768
				p = 0.327	*p* = 0.309
Physodic acid					***r* = -0.8165**
					***p* = 0.042**

Significant values are shown in bold (n=10).

We found significant differences in the total phenolics quantity in the bark between woody species (Fig. 3). Willow bark had the highest content of phenolic compounds, which was 3-times more than that of the ash bark. Comparison of Pearson correlation coefficients indicated that total extractable plant phenolics in host bark showed a strong positive correlation with physodalic acid, while a strong negative correlation with physodic acid content in lichen thalli (Table 1). We suppose that this contrary relationship is connected with different biosynthetic pathways of these lichen substances (43). It is well known, that phenolic compounds may affect the activity of enzymes, including those produced by plant-pathogenic fungi (2). Thus, bark phenolics may allelopathically interact with certain

enzymes, involved in the metabolism of lichen substances. Farther, the particular bark phenolic compounds, which qualitatively and quantitatively vary between tree species, may have variable effects on the phenolics in lichen thalli.

CONCLUSIONS

The concentrations of secondary metabolites in epiphytic lichens significantly vary with the host species and for certain compounds are correlated with bark parameters, including pH and phenolics level. These findings should be considered, when collecting and analysing the lichen samples from nature. The correlation described here between the total bark phenolics and *H. physodes* phenolics need to be clarified in farther studies, to determine the effects of particular host compounds and their concentration on the production of lichen secondary metabolites. Besides, other bark parameters not tested here (e.g. bark texture or moisture) can also be important. Therefore, further investigations are needed to understand the way in which substratum properties influence the pathways of lichen compounds biosynthesis.

ACKNOWLEDGEMENTS

We are grateful to Professor Zbigniew Lechowski (Jagiellonian University, Poland) for his valuable comments and discussion of this manuscript. The Faculty of Biochemistry, Biophysics and Biotechnology of Jagiellonian University is partner of the Leading National Research Centre (KNOW) supported by the Ministry of Science and Higher Education (Poland).

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