Chemical investigations of lichen biomass in *Usnea barbata*, *Cetraria islandica*, and *Xanthoria parietina* species

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Abstract. In this paper, determinations of secondary metabolites in three lichen species of biotechnological interest were achieved. The secondary metabolites from *Usnea barbata* (L.) Weber ex F. H. Wigg., *Cetraria islandica* (L.) Ach. and *Xanthoria parietina* (L.) Th. Fr. were determined by thin layer chromatography (TLC) in chloroform and acetone extracts. We used four elution systems in order to identify composition of extracts. The results obtained with different buffer systems revealed certain spots positions which can be identify by retention factor Rf compared with data from literature. Extract composition is different in acetone and chloroform respectively, and varies with the species. Most numerous compounds (15) from depsidone, depsones, depsides, dibenzofuranes, terpenoides and xanthones classes were noticed in *U. barbata*. For *C. islandica*, six compounds belonging to depsidones, xanthones, and anthraquinones and for *X. parietina* twelve different substances as depsidones, xanthones, and terpenoides were found. Some of these compounds as usnic acid, hypopsoromic and cryptostictic acid were commonly in all species. These results sustain the idea that elution systems used by us are suitable for identification of biosynthetic spectra in these lichen species.
Key Words: secondary metabolites, lichen components identification, biosynthesis, thin layer chromatography.

Introduction. The range of secondary metabolites identified in lichens is in continuous progress (over 500) (Brovko et al 2017). These are specific products and represent more than 40% of dry weight of the thallus (Purvis 2000); they are produced by the fungus symbiont, but they can be found in the gonidial cells too, proving, through this, that they are involved in the adjustment of the symbiosis (Ahmadjian 1993). The great progress recorded in the knowledge of the lichenic products in the second half of the 20th century is due to efficient methods of determining the chemical structure (thin layer chromatography - TLC, high performance thin layer chromatography - HPTLC, high performance liquid chromatography - HPLC) and to new synthesis techniques to confirm these structures (Culberson & Elix 1989; Huneck & Yoshimura 1996).

TLC and HPLC analysis of secondary metabolites has become fast an important tool in taxonomy and modern lichen phylogeny (Brodo 1986; Hawksworth 1988; Rogers 1989; Lumbsch 1998; Kranner et al 2003). The biological role of lichen substances have been reviewed by Lawrey (1986) and additional information on the ability of lichenic aromatic substances to protect against radiation can be found in Rikkinen (1995). Lichens may use secondary metabolites as chelating agents (Purvis et al 1987), like inhibitors of lichenicolous fungi (Lawrey 1995) and to avoid water saturation of the medulla (Armaleo 1993). Pharmaceutical companies are interested in using some of these compounds for their antifungal, antibacterial and antiviral properties. Lichen metabolites (aliphatic acids, depsides, depsidones, dibenzofuranes and derivatives of pulvinic acid) are used not only in medicine, but also in dyes industry, drugstores and agriculture (Rikkinen 1995; Elix 1996; Neamati et al 1997; Yamamoto et al 2002).

Socio-economic resonance of these lichen acids stimulated the researches regarding lichen biomass obtaining of biotechnological interest by *in vitro* culturing lichen thalli and homo/heterospecific artificial resynthesis, starting from aposymbiotic axenic cultures, of mycobiont and photobiont. Lichen biomass obtained through *in vitro* cultivation of thallus explants and artificial resynthesis have been already investigated (Voicu & Brezeanu 2008a, b; Cristian et al 2013; Cristian & Brezeanu 2013). In this paper we try to elaborate facile and feasible protocols for evaluate and secondary metabolites spectrum in these biotechnological interest species. For this purpose, appropriate eluting systems have been used for fast identification of biosynthesized compounds by thin layer chromatography.

Material and Method. The lichen biomass of *Usnea barbata* (L.) Weber ex F. H. Wigg., *Cetraria islandica* (L.) Ach., and *Xanthoria parietina* (L.) Th. Fr. was collected from forestry area Cumpatu from Sinaia between June to October 2008. The biologic material was taxonomical identified by Prof. N. Toma from Faculty of Biology, University of Bucharest. The processing and analysis methodology included lichen substances extractions, chromatographic separations of the compounds and identification reactions.

Preparation of lichen extracts. The lichen biomass of these three species has been subjected to extraction using the Soxlet extractor and a rotary evaporator for concentration according to Rai et al (2016) and Huneck & Yoshimura (1996). We used two types of solvents a non-polar solvent (chloroform) and a polar solvent (acetone).

Preparation of the chloroform extracts. Dry biomass, obtained by grinding with mortar and pestle, was extracted with chloroform 1:70 (g mL^{-1}) using the Soxlet extractor for 4h, at an interval of 24 hours. A light, opalescent, dark green extract was filtered on a cut filter at room temperature and a clear dark green/yellow filtrate was obtained.

The filtrate was concentrated in vacuum, at 35°C using a rotary evaporator. After concentration, the extract was dark brown and opalescent. It was kept cool (at 4°C) in the dark.

The acetone extraction. It contained the same steps: dry lichen material was covered with acetone and subjected for 3 days to extraction, using Soxlet extractor. The slightly opalescent, brownish-green acetone extract was filtered on a cut filter at room temperature, obtaining a clear, yellow-green filtrate. This was concentrated in vacuum, at 45°C using a rotary evaporator. After concentration, the obtained acetone extract becomes brownish-green colour. It was stored in the refrigerator at 4°C.

The test samples were scored as such:

- for chloroform extract (Soxlet): *Usnea barbata* (PC), *Cetraria islandica* (UC) and *Xanthoria parietina* (EC);

- for acetone extract (Soxlet): *Usnea barbata* (PA), *Cetraria islandica* (UA), *Xanthoria parietina* (EA), supplementary, for *Usnea barbata,* another two acetone extracts obtained by cold soaked (Pa) and powder resulting from chloroform extraction followed by precipitation with acetone (Pp), were tested.

Thin layer chromatography separation. The composition of the lichen extracts was determined by thin layer chromatography (TLC). For chromatographic separation were used Fluka silica gel 60 F_{254nm} pre-coated plates (layer thickness 0.2 and 0.1 mm). Several eluting systems have been tested for lichens extracts known for the best discrimination spots in lichen taxonomy according to the methods described in Lumbsch (2002). Therefore, for mobile phase, four solvents mixtures have been used: (A) toluene/dioxane/acetic acid (180/45/5), (C) toluene/acetic acid (170/30), (E) ciclohexane/ethyl acetate (75/25) and (G) toluene/ethyl acetate/formic acid (139/83/8).

For development of silica gel plates and visualization of the separated compounds, air-dried chromatographic plates were sprayed with 10 % sulphuric acid, heated at 110°C for 8 minutes for revelation of polyphenolic compounds (Schumm & Elix 2015).

The identification reactions used have been diversified according to Culberson & Elix (1989) for identification of the chemical nature of the secondary metabolites, as shown below:

A. Identification reactions used for gallic or hydrolysable tannins:

- treatment with hypochlorite resulting in a red-carmine/orange coloration for lichenic acids belong to the group of orchinal derived depsides;
- treatment with p-phenyldiamine giving a yellow coloration resulted in the presence of psoromic acid (of the group of despidones derived from β-orcinal).

B. Identification reactions used for catehic or non hydrolysable tannins:

- treatment with acetic acid-brominated water or Styassny reactive (formaldehyde/clorhidric acid conc. 2:1), resulting in a yellow precipitate.

C. Identification reactions used for benzofurane derivatives:

- treatment with hypochlorite resulting in a blue-green coloration in the presence of usnic acid (from *Usnea barbata*) or strepsiline (from *Cetraria islandica*).

D. Identification reactions of the anthraquinone derivatives:

- treatment with potassium resulting in a purple coloration in the presence of parietin (from *Xanthoria parietina*).

E. Identification reactions used for flavonoides:

- treatment with 5% NaOH resulted characteristic colouring: yellow, for flavones and flavonols, orange/red, for chalcones and aurones, and by heating flavanones appear coloured in red;
- treatment with 1% ferric chloride solution a characteristic stains resulted in dark - blue for 5-hydroxyflavones and 3',4',5'-hydroxyflavones and brownish for 3-hydroxy-flavones.

Additionally using induced fluorescence, the spots localization was achieved by exposure of Fluka silicagel $60F_{254nm}$ plates to UV light.

After staining the samples on the silicagel plate were processed with the WINMETABOLITES software (Elix et al 1988) for Rf compare with data from literature. The results of our extracts were correlated with specialty databases for rapid identification of lichen substances (Elix & Stocker-Wörgötter 2008; Elix 2014, 2016).

Results and Discussion. Extract components separation was more efficient by using Fluka silicagel $60F_{254nm}$-plates of 0.2 mm in place of 0.1 mm, a larger number of spots being separated and well delimited (Figure 1).

The different solvent mixtures allow a better discrimination of the components with high Rf values than with lower values.

The most lichenic components were separated by migration in A and C solvent systems. In C system, a successful separation of the spots along eluting area was observed. The most abundant in components are the extracts of *Usnea barbata* and *Xanthoria parietina*.

The E system led to the separation and highlighting of a smaller number of components, being more efficient for the substances with higher Rf value.

The G system was efficient for components with lower Rf values. In this elution system were determined the highest number of components in all types of extracts.

The chromatograms visualization in UV lights showed clearly spots position, but for the identification of the components, the specific colour reactions were required.

Thin layer chromatograms in UV or VIS showed a similar composition of *Usnea barbata* and *Xanthoria parietina*.

The results obtained after application of the four systems of solvents, were compared with the data existing in the literature (Elix et al 1984; Huneck 1991; Huneck & Yoshimura 1996; Elix & Stocker-Wörgötter 2008; Elix 2014; Rankovic & Kosanic 2015). A number of compounds considered representatives for the studied species, have been identified as it can be seen in Tables 1-3.

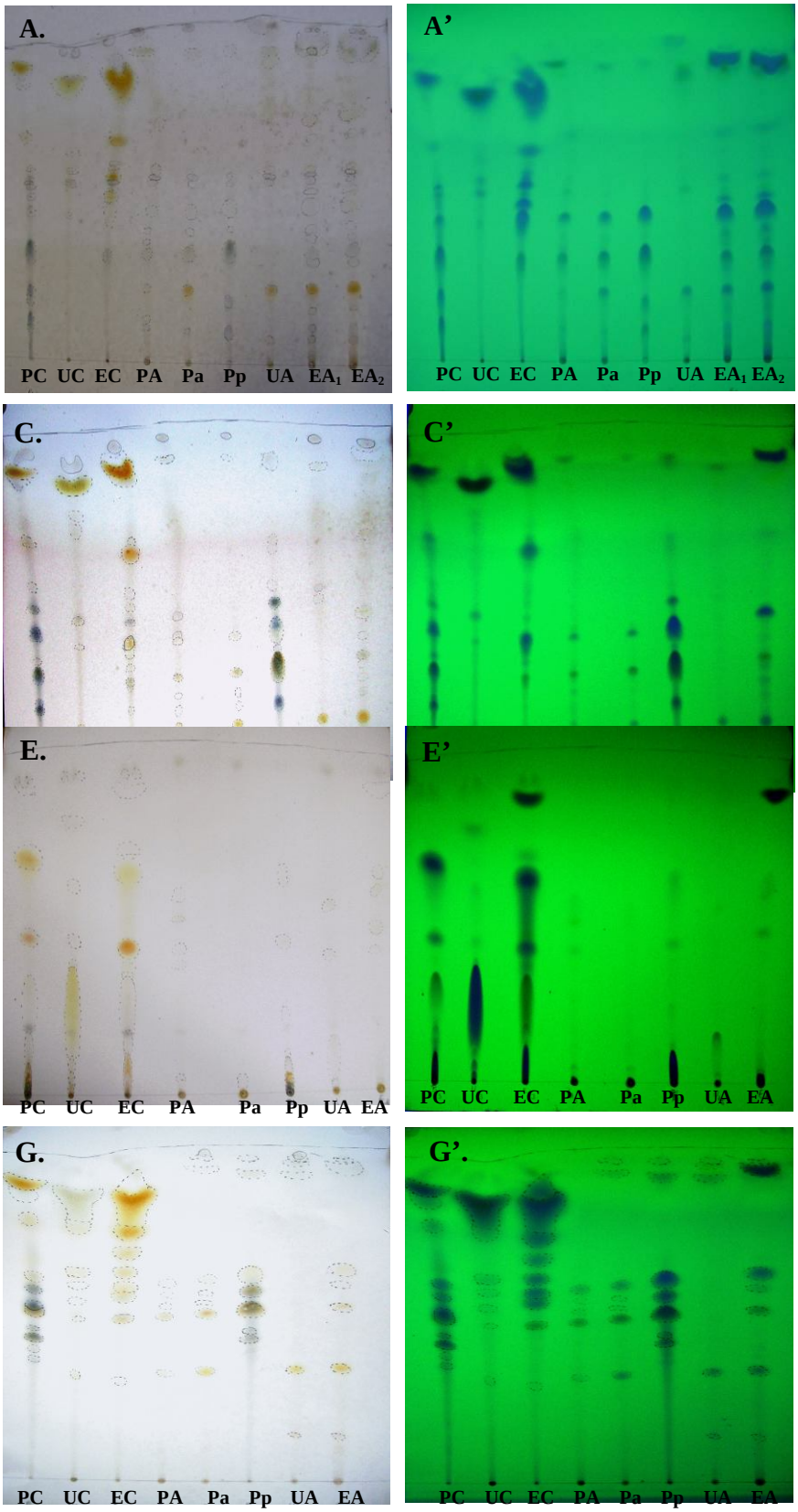

Figure 1. TLC plates developed with sulphuric acid after migration in all buffer systems: A, C, E, G respectively; and visualization in UV light: A', C', E' and G'.

Table 1

Identified compounds in *Usnea barbata* extracts

Substances	*Class*	*Estimated Rf value*			
		A	*C*	*E*	*G*
	Chloroform extract (PC)				
Hypopsoromic acid	Depsidones	0.33	0.17	-	-
9-methyl-4-hydroxypanarat	Dibenzofuranes	-	0.18	-	-
2,7-dichloronorlichexanthone	Xanthones	-	0.27	0.07	-
Hyperpicrolichenic acid	Depsones	-	0.39	-	-
5-dichlorovicanicin	Depsidones	0.54	0.41	-	0.18
Flebic acid	Terpenoids	0.45	0.47	0.25	0.5
superhyperpicrolichenic acid	Depsones	0.53	0.53	-	-
21-O-methylatranorin	Depsides	0.69	0.67	0.44	0.79
Usnic acid	Usnic acids	0.86	0.85	0.25	0.86
Thiomeline	Xanthones	0.91	0.88	0.66	0.9
	Acetone extract (PA)				
Cryptostictic acid	Depsidones	0.14	0.11	-	0.31
3-alfa hydroxybarbatic acid	Depsides	0.2	0.25	-	-
Hypopsoromic acid	Depsidones	0.31	0.16	-	-
5-chloronorlichexanthone	Xanthones	0.22	0.25	-	0.48
Secalonic acid	Anthraquinones	0.49	0.36	0.57	-

Table 2

Identified compounds in *Cetraria islandica* extracts

Substances	*Class*	*Estimated Rf value*			
		A	*C*	*E*	*G*
	Chloroform extract (PC)				
Leucoitic acid	Terpenoids	0.45	0.35	0.02	0.31
4-chloro-3-O-methylnorlichexanthone	Xanthones	0.54	0.54	0.35	-
Pannarin-methyleter	Depsidones	0.65	0.7	0.42	-
Usnic acid	Usnic acids	0.86	0.85	0.25	0.86
	Acetone extract (PA)				
Conprotocetraric acid	Depsidones	0.07	0.02	-	0.14
Cryptostictic acid	Depsidones	0.14	0.11	-	0.31

Table 3

Identified compounds in *Xanthoria parietina* extracts

Substances	*Class*	*Estimated Rf value*			
		A	*C*	*E*	*G*
	Chloroform extract (PC)				
Hypopsoromic acid	Depsidones	0.31	0.16	-	-
5-chloronorlichexanthone	Xanthones	0.44	0.24	-	0.47
Secalonic acid	Anthraquinones	0.49	0.36	0.57	-
2'-O-methylatranorin	Depsides	0.66	0.62	0.39	0.79
Usnic acid	Usnic acids	0.86	0.84	0.24	-
Thiomeline	Xanthones	0.91	0.87	0.61	0.83
	Acetone extract (PA)				
Conprotocetraric acid	Depsidones	0.07	0.02	-	0.14
Cryptostictic acid	Depsidones	0.14	0.11	-	0.31
Hypopsoromic acid	Depsidones	0.31	0.16	-	-
5-cloronorlichexanthone	Xanthones	0.22	0.25	-	0.48
3-alfa hydroxybarbatic acid	Depsides	0.2	0.25	-	-
Secalonic acid	Anthraquinones	0.49	0.36	0.57	-

Most of compounds identified by color reactions belong to the polyphenols class (depside, depsidone, dibenzofurans, xanthones), but also specific lichenic compounds belonging to the class of terpenoids or anthraquinones have been identified.

The two types of extracts are different regarding the chemical composition for all analyzed species, only the hypopsoromic acid was found in both chloroform and acetone extracts at *Usnea* and *Xanthoria*. Also, secalonic acid was detected in both extracts at *Xanthoria*, but not in *Usnea*.

Usnic acid is commonly in all chloroform extract of three species and cryptostictic acid is present in all acetone extracts. Acetone extracts of *Usnea* and *Xanthoria* are very similar in composition, except for conprocetraric acid which found in *Xanthoria*, but not in *Usnea*. The fewest compounds were identified for *Cetraria islandica*.

Although the compounds identified by color reactions and Rf retention factor in the TLC databases are specific to metabolites of these species, their presence remains to be confirmed by using of standard compounds.

Lichen biomass production rich in active principles of biotechnological interest can be improved by *in vitro* culture. We already developed protocols for obtaining of simbiotrophic formations to *Cetraria islandica* and *Usnea barbata*, and micobiont production to *Xanthoria parietina*. The production of secondary metabolites can be significantly improved through variation of culture conditions (phytohormones addition, variations of physical parameters as temperature, light intensity or photoperiod, and different treatments with biotic and abiotic elicitors). All of these results are sufficient arguments to hope in the possibility of obtaining *in vitro* lichen biomass which produce certain metabolites of biotechnological interest.

Conclusions. The study results as well reporting them to literature led us to the conclusion that compounds synthesized by lichens continue to be of great interest for their unique chemistry and biotechnological potential.

The most commonly applied technique, namely thin-layer chromatography proved to be a rapid and efficient method for screening of biosynthesized compounds for lichens. This was possible due to existence of an extended database with lichen metabolites identified by TLC employed traditional in taxonomical studies.

These three studied species are of great importance for pharmacological industry because of their metabolites which have been use for long time in pharmacognosy.

Acknowledgements. The study was funded by project no. RO1567-IBB06/2010-2016 from the Institute of Biology of Romanian Academy, Bucharest.

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Received: 29 December 2018. Accepted: 16 February 2019. Published online: 30 March 2019.
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How to cite this article:
Voicu D. M. F., Mitoi M. E., Gavriloaie C., Helepciuc F. E., Toma N., 2019 Chemical investigations of lichen biomass in *Usnea barbata*, *Cetraria islandica*, and *Xanthoria parietina* species. ELBA Bioflux 11(1):1-8.