Cladonia uncialis as a valuable raw material of biosynthetic compounds against clinical strains of bacteria and fungi*

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Cladonia uncialis is a lichen species with confirmed antibacterial activity and whose genome has been recently sequenced, enabling first attempts in its functional characterization. In this work, we investigated activity of the C. uncialis acetone extract (CUE) and usnic acid (UA) enantiomers against ten clinical microbial strains causing skin infections. The results showed that CUE, containing (–)-UA and squamatic acid, assayed at the same concentrations as UA, was noticeably more active than (–)-UA alone, in its pure form. The studied CUE displayed an activity that was comparable to that of (+)-UA observed for Staphylococcus epidermidis and Enterococcus faecium (18–24 mm zone of growth inhibition), but did not display any activity against fungal strains. The CUE demonstrated low cytotoxicity against HaCaT cells, in comparison to UA enantiomers, which is important for its therapeutic use. Results of the antioxidant assay (DPPH) indicated low antioxidant activity (IC_{50}=200 μg/mL) of CUE, while the total phenolic content was 70.36 mg Gallic Acid Equivalent/g of the dry extract.

Key words: lichen metabolites, lichen extract, antibacterial activity, cytotoxicity, HaCaT, antioxidant activity

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INTRODUCTION

Lichen species produce a wide range of secondary metabolites to increase their survival fitness under extreme conditions. Numerous studies had shown that lichen micro-ecosystems produce substances that demonstrate multidirectional biological activity and thus could be used in pharmacology (Lücking et al., 2016). About 50% (Candan et al., 2007) of all 19000 lichen species known to date (Abdel-Hamied et al., 2016) contain antibacterial substances that could be used against antibiotic-resistant bacterial strains in treatment of wounds, skin infections, and upper respiratory and gastrointestinal symptoms. The most known lichenic compound is usnic acid (UA), existing in two enantiomeric forms of which (+)-UA occurs more frequently (Cetin et al., 2008). Results of research carried out so far indicate a high antibacterial activity of UA, mainly against Gram-positive strains (Galanty et al., 2019). Most of studies on antibacterial activity of UA were focused on its (+) enantiomer, which usually demonstrated an increased activity in comparison to its (–) form (Galanty et al., 2012). In addition to antibacterial properties, UA has also antioxidant, anti-proliferative, antiprotrozoal, larvicidal and insecticidal, antifungal, antiviral, algicidal, anti-inflammatory, pain-relieving and antipyretic properties, as has been demonstrated by *in vivo* and *in vitro* studies (Alahmadi, 2017).

Cladonia uncialis L. Weber ex F.H. Wigg., (order Cladonaceae), is a fruitaceous lichen, growing on humus or sandy soil, mainly in pinewood forests, on moors, dunes or roadside. This species is found on all continents except Antarctica, especially in the Northern Hemisphere. In Poland, it is common throughout the country (Purvis et al., 1992; Wójcik, 2007). C. uncialis is known for its biosynthesis of (–)-UA, hardly observed in other lichen species (Abdel-Hameed et al., 2016). Recently, the C. uncialis genome has been sequenced, which allowed for its functional genomic analysis (Bertrand et al., 2018a). A number of biologically active secondary metabolites have been characterized via a homology mapping approach (Bertrand et al., 2018a; Bertrand et al., 2018b), including the well-studied aromatic polyketides (Bertrand et al., 2019). Nevertheless, functional analysis of C. uncialis and other lichens involving heterologous expression or gene knockout experiments is still challenging due to their slow rate of growth, obligate symbioses and other difficulties in establishing their cultures (Grube et al., 2014; Bertrand et al., 2019; Cimmino et al., 2019). What is more, differences in cultivation conditions have impact on the extract composition (Bjerke et al., 2005; Brisdelli et al., 2013; Studzińska-Sroka et al., 2015). Consequently, there is still a noticeable disproportion between known biosynthetic gene clusters and characterized secondary metabolites of C. uncialis and other lichens (Bertrand et al., 2018a). To date, the genome sequencing of C. uncialis has revealed (Bertrand et al., 2018a) presence of 48 biosynthetic gene clusters, including type I and III polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS), hybrid PKS-NRPS and a terepne synthase. The C. uncialis genome was proposed to include enzymes for grayanic acid, patulin and betaamone biosynthesis, and the latter
two were not found in other lichen species (Bertrand et al., 2018b). A recent genome mining study of other lichen species, *Exorexia praenustri* and *Pseudorevia furfuracea*, reported 80 biosynthetic gene clusters in the first one and 51 in the latter (Calchera et al., 2019) that also included polyketide synthases, non-ribosomal peptide synthetases and terpene synthases.

The thallus of *C. uncialis* contains (−)-UA, as well as squamatic and thamnolic acids (Purvis et al., 1992), but the biological activity of its extracts is almost unknown. The antibacterial activity of *C. uncialis* extracts has been proven for the first time in our previous studies, in which we demonstrated a high activity of the *C. uncialis* acetone extract (CUE) on methillin-resistant *Staphylococcus aureus* (MRSA) strains (Studzińska-Sroka et al., 2019). This interesting finding, as well as previous information about antimicrobial activity of UA (Galanty et al., 2017; Galanty et al., 2019), prompted us to expand the study on the activity of CUE against clinical pathogenic strains responsible for skin and wound infections, which demonstrate resistance to antibiotics. In our research we also compared the cytotoxic effect of the tested substances by employing in vitro studies with human keratinocyte cell cultures (HaCaT). Although the cytotoxic properties of (+)-UA on HaCaT cells were previously studied, the respective research involving CUE and (−)-UA has not been conducted yet (Brisdelli et al., 2013). We determined the CUE composition using NMR and MS, while the quantity of (−)-UA in CUE was determined with HPLC. The content of polyphenols (TPC) was evaluated with the Folin-Ciocalteu (FC) analysis. The antioxidant activity of (−)-UA and CUE was also investigated.

**MATERIALS AND METHODS**

**Plant material.** The *C. uncialis* (L.) Weber ex F.H. Wigg. lichen was manually collected in September 2014 in Jastrzębsko Stare, Poland, and authenticated by dr Daria Zarabska-Bożejewicz (The Institute for Agricultural and Forest Environment of Polish Academy of Sciences in Poznan). A voucher specimen (ES 2014.007) has been deposited in the herbarium of the Department of Pharmacognosy at Poznan University of Medical Sciences.

**General.** Acetone, dimethylsulphoxide (DMSO), methanol, sodium carbonate and HPLC grade: water, chloroform and methanol, were purchased from POCH, Gliwice, Poland; HPLC grade: 85% phosphoric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the reference compounds; gallic acid, vitamin C and BHA were from Sigma Aldrich, USA. Folin-Ciocalteu’s phenol reagent, was from Merck, Germany; Usnic acids: (−)-usnic acid and (+)-usnic acid came from the compounds’ collection of the Pharmacognosy Department (their identity, optical rotation and purity were assessed by: UV, 1H NMR, 13C NMR, MS, and polarimetric analysis).

**Preparation of extracts.** Air-dried and coarsely ground lichen thalli (19 g) were extracted four times with 200 mL of acetone (800 mL total) in a ultrasonic bath (Elmasonic S 180H, Elma, Germany) at 30-35 °C for 30 min. The extracts were filtered using Whatman filter paper No. 1 and then evaporated under reduced pressure at 50°C, yielding 201.4 mg of solid residue.

**Determination of the total phenolic content.** TPC was determined using the Folin-Ciocalteu method described by Gawron-Gzella and others (Gawron-Gzella et al., 2016), with modifications. Briefly, 0.1 mL of CUE dissolved in DMSO (10 mg/mL) was mixed with 7.4 mL of distilled water and with 0.5 mL of the Folin-Ciocalteu reagent. The mixture was shaken vigorously and 2.0 mL of 20% (w/v) sodium carbonate solution was added after 1 min. Next, the samples were incubated for 30 min in the dark, at room temperature. The absorbance was measured at 760 nm (UV/VIS Lambda 35 PerkinElmer). Total polyphenol concentration was calculated using gallic acid (0.02-0.08 mg/10 mL of sample) as a standard for a calibration curve (y=0.1016x – 0.0029, R²=0.9993). Total concentration of phenolic compounds in the extract was expressed as mg of gallic acid equivalent/g of the dry extract. The values are expressed as the mean of six replications ± S.D.

**Quantification of (−)-usnic acid in the extract.** The quantity of (−)-UA in *C. uncialis* acetone extract (CUE) was examined by HPLC as described previously (Studzińska-Sroka et al., 2015), with water and 1% 0.1N H₃PO₄ (v/v) (A) and methanol (B), A:B 15:85 as the mobile phase. Detection was carried out with a PDA detector set at 250 nm. To prepare the stock solution, 5.0 mg of (−)-UA was dissolved in 5.0 mL of chloroform. In order to obtain calibration curves, appropriate volumes of the stock solution were transferred to volumetric flasks and diluted with the same solvent to give standard solutions of the following concentrations: 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/mL (y=963.73x – 16.051; R²=0.9986). Each concentration was analysed in triplicate under conditions described above. In order to analyse the samples, 5.0 mg of the tested extract was dissolved in 5.0 mL of chloroform. The results were reported as the arithmetic mean ± S.D. for three replications.

**Quantification of (−)-usnic acid in the extract.** Identification of main compounds in CUE was carried out by NMR (700 MHz NMR spectrometer Bruker Avance III). Spectra were recorded after dissolving the substance in acetone-d₆ and over TMS. ESI-MS data were acquired with Waters Maldi Q-Tof Premier mass spectrometer.

**Determination of antibacterial activity.** In this study, strains mostly responsible for skin infections were employed: clinical strains of Gram-positive bacteria - staphylococci (*Staphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus haemolyticus*), enterococci (*Enterococcus faecalis and Enterococcus faecium*), streptococci (*Streptococcus pyogenes*), Gram-negative bacilli (*Escherichia coli* and *Pseudomonas aeruginosa*), and yeast-like fungi (*Candida albicans* and *Candida glabrata*). The isolated strains were tested for their antibiotic sensitivity and the following culture media were used: Mueller Hinton Agar (OXOID), Mueller Hinton Agar with horse blood (OXOID) for bacterial strains, and RPMI agar (BioMaxima) for yeast-like fungi. Disc diffusion method was used to determine the antimicrobial activity, and therefore the results were expressed as the diameter of the zone of growth inhibition of the tested microorganisms by the tested substances. For the antimicrobial study, solutions of the acetone extract, as well as (−)-UA and (+)-UA in DMSO, were prepared (10 mg/mL). Blank sterile discs (Ø=6 mm; OXOID) were placed with slight pressure on inoculated media and impregnated with 10 μL of the tested substances to obtain a content of 100 μg of the extract or usnic acids in the disc. The Petri Dishes were incubated for 24 h for bacteria and 48 h for fungi, and the zone of inhibition was measured. Standard antibiotics’ dishes for the tested microorganisms for Gram-positive bacteria (penicillin, amoxicillin/clavulanic acid, vancomycin, erythromycin, and clindamycin), to compare the inhibition zones around the antibiotic disc, were used as positive con-
Viability assay. Spontaneously immortalized HaCaT keratinocytes (Cell Lines Service, Germany) were grown in a high-glucose DMEM medium (BioWest, France), supplemented with 10% FBS (EURLx, Poland), and 1% antibiotics solution (penicillin and streptomycin, BioWest, France), in a humidified incubator in the atmosphere of 5% CO₂. Stock solutions of the dry extract, (–)-UA and (+)-UA samples were prepared in DMSO. The effect of the solutions on the viability of HaCaT cells was assessed by using the MTT assay. Cells were seeded (2×10⁴ cells/well) in a 96-well plate and preincubated for ~24 h. Next, fresh medium containing varying concentrations of the tested substances (1–50 µg/mL) was added into the wells and the cells were incubated for subsequent 72 h. Afterwards, the cells were washed with PBS buffer and a fresh complete medium containing 0.5 mg/mL of MTT salt (Sigma, USA) was added. After a 4-hour incubation, formazan crystals were dissolved in acidic isopropanol and the absorbance was measured at 570 nm and 690 nm. The assay was repeated three times with five measurements per assay. The results were calculated as percentage viability compared to control cells only treated with the vehicle.

DPPH assay. The free radical scavenging capacity of the acetone extract from Cladonia uncialis was determined using the DPPH method described by Annegowda et al. (2010). Briefly, 0.1 mL of CUE, (–)-UA or (+)-UA, dissolved in DMSO (6.0–0.1875 mg/mL) was added to 2.9 mL of DPPH solution (0.1 mM in methanol; the final assay concentrations were 200.0–6.25 µg/mL). The reaction mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm against a blank (2.9 mL of methanol + 0.1 mL of DMSO) (UV/VIS Lambda 35 PerkinElmer). The control was a mixture of 2.9 mL of DPPH solution and 0.1 mL of DMSO. Vitamin C and BHA were used as the referring substances. The following equation was used to calculate concentration of the DPPH radicals:

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\text{DPPH scavenging activity} (\%) = \frac{A_0 - A_t}{A_0} \times 100\%
\]

where A₀ is the absorbance of the control and Aₜ is the absorbance of the reaction mixture or standards. The IC₅₀ values were calculated as the concentration required to inhibit 50% of DPPH radical. The values have been expressed as the mean of six replications. Homology modelling of C. uncialis polyketide synthase

A homology model of C. uncialis non-reducing polyketide synthase was constructed using the C. nicotianae crystal structure (Herbst et al., 2018) (PDB id: 6FJI), comprising of three domains: SAT, KS and MAT. This template, of the highest sequence overlap with the target, was selected by BLAST (Madden et al., 1996) from PDB (E-value equal to 3e-104, sequence identity 26.53% and sequence coverage 46%). Only the KS domain model of the polyketide synthase (Fig. 1) was constructed using the BLAST-derived sequence alignment, MODELLER (Webb et al., 2016), and a previously developed procedure (Latek, 2017).

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) and Microsoft Excel 2013 software (Microsoft, Redmond, WA). The median effect concentrations (IC₅₀ values) were determined using a concentration-response curve.

RESULTS AND DISCUSSION

Phytochemical characterisation of the extract

Chemical composition of CUE (201.4 mg, yield 1.06%), examined by 1H NMR and MS, confirmed that its main compounds were UA and squamatic acid (result presented in Fig. 2). Presence of thamnolic acid described in the C. uncialis thallus was not confirmed with sufficient certainty using the applied procedure. The content of (–)-UA (52.52%±0.28), in the extract analysed by HPLC, was higher than in our previous study (28.40%) (Studzińska-Sroka et al., 2015). This difference, as well as the lack of thamnolic acid in the studied extract, could result from various atmospheric conditions during the vegetation period of the lichen (Bierke et al., 2005; Millot et al., 2007; Neupane et al., 2017). The results of FC analysis showed TPC equal to 70.36 ± 1.42 mg GAE/g of the dry extract, which is in accordance with data on TPC in other Cladonia species (Mitrovic et al., 2011).

Antimicrobial activity

The emerging problem of bacterial resistance to existing antibiotics prompted research of new antibacterial agents that were derived from natural substances. Our study was aimed at evaluating the antimicrobial activity of CUE and UA enantiomers against eight clinical bacterial and two fungal strains, mainly responsible for skin infections and post-operative wounds. The obtained results demonstrated an increased activity of the tested substances against selected Gram-positive microorganisms, with no impact on Gram-negative strains (Table 1). The highest growth inhibitory zone (GIZ) was obtained for coagulase-negative staphylococci (CNS) (S. epidermidis, S. baemolyticus) and enterococci (E. faecalis, E. faecium). S. aureus and S. pyogenes were also affected, but to a lesser extent. In the case of enterococci and CNS (S. epidermidis, S. baemolyticus), GIZ was almost the same between (+)-UA and CUE, but smaller for (–)-UA. For S. aureus, no...
major differences between the tested substances were observed. For S. pyogenes, GIZ for (+)-UA was the smallest. It is widely known that therapeutic effect of chiral drugs may differ for the particular enantiomers (McConathy et al., 2003). Our results show that (+)-UA demonstrated the highest antibacterial activity against 5 out of 6 examined strains of Gram-positive bacteria, while (–)-UA acted more strongly only on S. pyogenes. The mechanism of antibacterial action of usnic acid was previously described. Namely, UA induces inhibition of RNA and DNA synthesis in Gram-positive bacteria (Bacillus subtilis and S. aureus), while in E. coli it does not inhibit production of any macromolecules (DNA, RNA, and proteins) (Maciąg-Dorszyńska et al., 2014). This may explain the effect of the extract and usnic acid on the individual microbial strains involved in this study. Interestingly, our results showed that CUE, containing 52.52% of (–)-UA, demonstrated a higher activity in comparison to pure (–)-UA used at the same concentration. In our previous study, we observed that squamatic acid,
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Figure 3. The effect of lichen-derived substances on the viability of HaCaT cells. Mean values ± S.E.M. obtained from three independent experiments are shown.

present in CUE, had no antibacterial effect against S. aureus (Studzińska-Sroka et al., 2015), while thamnolic acid, whose presence in this specie was described in the literature, demonstrated only a very weak activity: MIC 200–400 µg/mL, for Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi (Cankılıç et al., 2017). Based on the above, we suppose that the extract should contain low quantities of other, unidentified compounds that were responsible for its antibacterial activity and/or the specific synergism of its action. In this study, we observed a high effectiveness of CUE against Gram-positive bacteria: S. bueniolicus, E. faecalis and E. faecium that are known for their resistance to commonly used antibacterial drugs (Amyes et al., 2007; Fredheim et al., 2009). In this context, our results for the tested lichen-derived substances, especially CUE, may have future therapeutic applications as antibacterial agents for antibiotic-resistant strains. Lack of the antifungal activity of the lichen extract and its compounds has often been described (Yang et al., 1999).

Cell viability

Keratinocytes (HaCaT) are a good in vitro model of epidermis. Other studies indicated that (+)-UA had a strong cytotoxic activity against keratinocytes (EC$_{50}$ 35 µM and 75 µM, in crystal violet or neutral red uptake assays, respectively) (Burlando et al., 2009). Our results show that (+)-UA was more cytotoxic in comparison to (–)-UA and CUE (at a concentration of 5 µg/mL, the viability of HaCaT cells was: 88.3%, 61.4% and 46.9% for CUE, (–)-UA and (+)-UA, respectively) and this effect was dose-dependent (Fig. 3). Differences in cytotoxicity among enantiomers of UA have been already described in the literature (Galanty et al., 2019), but numerous studies questioned using cancer cell lines for this purpose. Namely, it was shown that the cancer cells were usually more sensitive to (–)-UA (L1210, Du145, K562, breast cancer, Capan-2, breast adenocarcinoma) (Bazin et al., 2008; Einarsdóttir et al., 2010; Ebrahim et al., 2017), whereas (+)-UA was usually more toxic to V79, A549 (Koparal et al., 2006). Keratinocyte cells (HaCaT) were supposed to be a more standard biological system for testing CUE cytotoxicity but there has been no experimental data confirming this hypothesis and, to our knowledge, our study is the first one to demonstrate this. Our results indicate the safety of using CUE as an antibacterial agent and suggest that using it in antimicrobial ointments could be beneficial.

Antioxidant activity

So far, no studies have been published on the antioxidant activity of C. uncialis and (–)-UA. The results of our DPPH analysis showed that CUE, (–)-UA and (+)-UA had low antioxidant activity (IC$_{50}$> 200 µg/mL), at 200 µg/mL the ability to scavenge DPPH was 9.8%, 13.2% and 14.0% for CUE, (–)-UA and (+)-UA, respectively. The observed low antioxidant activity of (+)-UA is consistent with previous data (IC$_{50}$=691 µg/mL) described by Ananthi and others (Ananthi et al., 2015). Moreover, Brisdelli and others (Brisdelli et al., 2013) demonstrated that usnic acid at the concentration of 0.8 mM, did not show a significant ability to reduce the concentration of the DPPH radical. Taking into account results of the above research, it can be assumed that the low antioxidant activity of CUE shown in our DPPH analysis, is probably due to a significant UA content. Despite the poor ability to scavenge the DPPH radical, UA displayed antioxi-
Molecular description of the KS domain of non-reducing polyketide synthase

*C. uncialis* methylphloracetophenone synthase (MPAS), like other polyketide synthases (PKS) (Herbst et al., 2018), is a microbial multidomain enzyme that catalyzes polyketide biosynthesis in various species. The first step of polyketide synthesis involves loading/condensing a substrate onto an integral acyl carrier protein (ACP) and then transferring it to the ketosynthase (KS) domain interacting with the starter unit acyltransferase (SAT) and malonyl-CoA-specific aetyltransferase (MAT) domains. Other domains of *C. uncialis* MPAS (UniProt entry: A0A0R8YWJ7) include the methyltransferase (CMeT) and Claisen cyclase (CLC) domains. Polyketide synthesis begins at the binding site of KS domain with a conserved Cys547 residue (see Fig. 4). The active site tunnel of *C. uncialis* KS is similar to the active site of *Cercospora nicotianae* KS, for which the SAT-KS-MAT complex structure is available in PDB (Herbst et al., 2018). There is, however, a difference in one active site residue (Thr instead of Cys) that is close to the active site Cys547 (see Fig. 4). The first stage of the polyketide synthesis involves forming a thioester between the carboxyl group of the polyketide starter and SH of Cys547, and further elongation of the polyketide chain (MAT domain) and cyclization (CLC domain) (Herbst et al., 2018). The resulting intermediate product of MPAS is methylphloracetophenone that subsequently undergoes oxidative dimerization to usnic acid which is carried out by the methylphloracetophenone oxidase (MPAO) (Abdel-Hameed et al., 2016).

CONCLUSIONS

Based on the results of microbiological tests, the actetone extract from *C. uncialis* may be considered as a source of interesting lignen substances, helpful in treatment of skin infections caused by antibiotic-resistant bacterial strains. It should be emphasized that CUE is less toxic and at the same time its antibacterial activity is stronger than pure (+)-UA, used, e.g. as a antibacterial agent in topical formulations (Bruno et al., 2013; Francolini et al., 2018). The biosynthesis potential of *C. uncialis* and other lichens is still poorly explored due to the lack of any experimental evidence for definite linking biosynthetic gene clusters with secondary metabolites. Only recently, first attempts in the functional genome annotation have been carried out (Bertrand et al., 2018a; Calchera et al., 2019). Nevertheless, their importance as a natural source of polyketides from which a number of pharmacologically active substances is derived (Helfrich et al., 2014), would increase the intensity of ongoing research.

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**Conflicts of Interest**

The authors declare no conflict of interest.

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