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RESEARCH ARTICLE

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Antimelanoma Potential of *Cladonia mitis* Acetone Extracts – Comparative *in Vitro* Studies in Relation to Usnic Acid Content

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In this study, the cytotoxic activity of acetone extracts of *Cladonia mitis* was assessed with respect to the content of usnic acid, a secondary metabolite commonly present in this species. Following quantitative HPLC analysis of the extracts, usnic acid was isolated by preparative chromatography. The study of cytotoxic activity was performed using the MTT test on three melanoma cell lines – HTB140, A375 and WM793. The selectivity of action was also assessed by comparing the effect towards normal human keratinocytes HaCaT. The results showed a dose-dependent cytotoxic activity of the extracts tested and usnic acid itself, but no relationship was found between the content of usnic acid and the activity of the extracts. Furthermore, the extracts showed varied, but rather low anti-tyrosinase activity. Other *in vitro* and *in vivo* studies are necessary to demonstrate that *C. mitis* extracts may be useful in the adjuvant external treatment of skin melanoma.

Keywords: *Cladonia mitis*, usnic acid, melanoma, tyrosinase.

Introduction

Lichens, as symbiotic organisms, consist of a mycobiont, a fungus, and a photobiont, usually green algae and/or cyanobacteria, responsible for the photosynthesis process. The type of mycobiont, which is a host partner for the photobiont, determines the morphological characteristics of the species and the taxonomic name of the lichen.[1] This cooperation results in the unique chemical composition of lichens, which is translated into their interesting biological effects. Among many different activities of lichens, their antibacterial, anti-inflammatory, and cytotoxic properties seem to be most promising, and their external use, for disinfecting wounds, in skin infections and ulcers, is widely described.[2]

The lichen genus *Cladonia* contains more than 60 species, the extracts of which have been extensively studied for various biological activities.[3–5] Among them, *Cladonia mitis* (syn. *Cladina mitis* (Sandst.) Mong., *Cladina arbuscula* subsp. *mitis* (Sandst.) Burgaz), *Cladoniaceae*,[6] is one of the lesser examined species. Data on its chemical content include reports on the presence of atranorin, fumarprotocetraric acid, rangiformic acid, psoromic acid and usnic acid.[7] Though results of quantitative studies on usnic acid in *C. mitis* are scarce,[8–10] some data indicate that this lichen species might be a novel, rich source of the compound, with its amount up to 2.2%.[10]

Melanoma is a skin cancer that is the most malignant of all known tumors, the disease is fatal in 80% of patients.[11] The effectiveness of antimelanoma therapy could be decreased by concomitant symptoms, such as inflammation or overexpression of tyrosinase, a key enzyme in melanin synthesis, resulting in the progression of the disease. Usnic acid, which shows significant cytotoxic, photoprotective and anti-inflammatory activity, may be a potential candidate for

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external drug in future melanoma treatment. Our previous work showed interesting antimelanoma and anti-inflammatory potential of usnic acid.[12] However, taking into account a phenomenon commonly observed in phytotherapy, that the therapeutic potential of complex extracts in some cases is comparable to or even greater than the effects observed for isolated, predominant compounds, we expanded our study. Therefore, our aim was to verify whether *Cladonia mitis* extracts, quantified in usnic acid, may also have the antimelanoma potential themselves and, if so, is the presence of usnic acid crucial for this action. Our previous experiments indicated that acetone was the most effective solvent for usnic acid extraction,[13] thus, in the present study, the acetone extracts of *C. mitis* were taken into account. We compared the activity of extracts differing in usnic acid content, using an *in vitro* model, consisting of three human melanoma cell lines: HTB140, A375, and WM793, and normal HaCaT skin keratinocytes, with the latter cells serving as selectivity indicators. The model was also completed with an anti-tyrosinase assay, to better assess the anti-melanoma potential of the *C. mitis* extracts.

Table 1. Usnic acid content in *Cladonia mitis* (mg/g dry wt.) samples, collected at different localizations (the detailed information is previously described[10]).

Sample	Country of collection	Usnic acid content [mg/g dry wt.]*
N1	Norway	17.69 ± 0.90
S1	Sweden	12.97 ± 0.60
S2		16.69 ± 0.80
S3		10.18 ± 0.44
S4		11.02 ± 0.64
S5		18.29 ± 1.33
S6		17.41 ± 0.76
S7		10.55 ± 0.74
S8		8.24 ± 0.35
S9		9.32 ± 0.67
S10		11.92 ± 0.66
S11		21.58 ± 2.23
P2	Poland	5.75 ± 0.62
P3		4.52 ± 0.54
P4		8.58 ± 0.68
P5		9.48 ± 0.30
P6		11.00 ± 0.73
P7		8.56 ± 0.61

*The mean content of usnic acid ± standard deviation ($n = 3$).

Results and Discussion

Following the prospective results obtained for usnic acid in our previous studies on normal and cancer skin cells,[12,14] we decided to determine the cytotoxic potential of *Cladonia mitis* acetone extracts and to compare it with the impact of usnic acid alone. In the present study we used 19 *Cladonia mitis* samples, collected along the North-South European transect, in which the usnic acid content was determined in our previous work.[10] To compare the activity of the extracts and usnic acid, we decided to isolate the compound from the sample with its highest content, namely S11 (see *Table 1*).

Chromatographic separation of the S11 extract on silica gel resulted in isolation of 49.4 mg of a yellow solid substance, of high purity (95.73 %), with retention time and UV spectrum consistent with standard usnic acid. The next stage of the work focused on the evaluation of the cytotoxic activity of *C. mitis* extracts and isolated usnic acid on human cancer and normal skin cells. Three melanoma cell lines, differing in metastatic potential, were used to mimic the complex nature of the real tumor. To verify the selectivity of the extracts tested, normal skin keratinocytes HaCaT were also included in the study. A wide range of concentrations (10–100 μg/mL) and the effect on the cells was assessed after 24 h of incubation. The results obtained are presented for the lowest and two highest concentrations tested (10, 50 and 100 μg/mL) in *Figure 1*, and the IC_{50} values are presented in *Table 2*.

For all examined melanoma cell lines, a dose-dependent decrease in the number of living cells was observed for most of the extracts. Melanoma A375 cells were most susceptible to the tested extracts, with

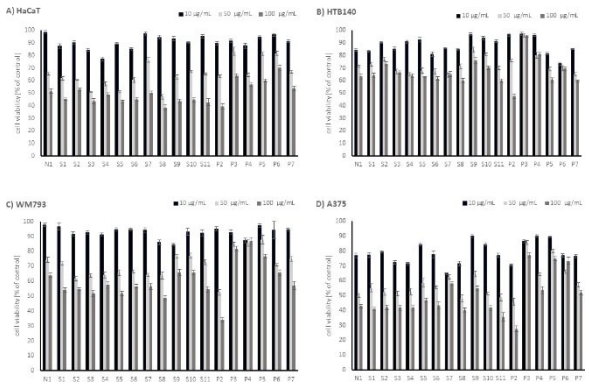

Figure 1. The effect of *Cladonia mitis* extracts on viability of skin keratinocytes HaCaT (A), melanoma HTB140 (B), WM793 (C), and A375 (D) cells.

Table 2. The effect of *Cladonia mitis* extracts and usnic acid on the viability of human melanoma WM793, A375, and HTB140 cells and normal skin keratinocytes HaCaT, expressed as IC_{50} (the concentrations that induced a 50% decrease in cell viability after 24 h of treatment).

	IC_{50} [μg/mL]			
	WM793	A375	HTB140	HaCaT
N1	>100	55.73	>100	70.01
S1	>100	61.72	>100	>100
S2	>100	52.73	>100	>100
S3	>100	50.86	>100	49.01
S4	>100	57.66	>100	>100
S5	>100	78.26	>100	55.72
S6	>100	64.78	>100	78.35
S8	>100	41.14	>100	48.7
S9	>100	>100	>100	64.77
S10	>100	53.18	>100	75.3
S11	>100	50.75	>100	74.6
P2	52.11	42.32	91.19	77.04
USNIC ACID	25.65	30.25	13.98	25.78
DOXORUBICIN	>100	1.59	3.77	4.68

For extracts S7, P3–P7 IC_{50} exceeded the highest concentration examined.

the S8, S11, P2 extracts the most active (*Figure 1D*, *Table 2*), but the differences between these three extracts were not statistically significant ($p > 0.05$). For WM793 and HTB140 melanoma cells, the strongest activity was demonstrated by extract P2 (*Figure 1C* and *1B*, *Table 2*). The extracts tested showed moderate selectivity of action, which was observed especially at lower concentrations, up to 40 μg/mL (*Figure 1A*). At a concentration of 50 and 100 μg/mL, the activity of the extracts was in some cases higher against normal HaCaT keratinocytes than against cancer cells. It is also important to mention that the antimelanoma profile of the *C. mitis* extracts differed from that of usnic acid. The compound itself showed the strongest activity against HTB140 cells, with A375 cells being the most resistant, while the cytotoxic impact of the extracts was just the opposite. Furthermore, no correlation was found between the activity of the extracts tested and the content of usnic acid, which means that the cytotoxic activity of the extracts studied may result from the presence of other secondary metabolites in *C. mitis* species, such as atranorin, fumarprotocetraric acid, rangiformic acid, or psoromic acid.[7] However, our analysis indicated the absence of the compounds mentioned above in the tested extracts, thus, further studies are necessary to shed more light on this question. It should be noted that so far only a few studies have described the cytotoxic activity of *C. mitis* extracts and all of them concerned murine and human leukemia cells.[8,15–16] The described activity of the methanolic extracts from *C. mitis* was low, with IC_{50} 180.2 and 418.4 μg/mL against HL-60/MX2 cells,[8] while in another study, the hexane extract strongly decreased the viability of CCRF-CEM leukemia cells (IC_{50} 6.63 μg/mL).[16] Thus, our study has not only expanded existing data on the cytotoxic potential of this species, but also provided results with respect to melanoma cells for the first time.

Tyrosinase is an important enzyme in controlling melanin synthesis. Excessive expression of tyrosinase in melanoma cells may decrease their sensitivity to radio- and chemotherapy.[17] Therefore, in the last step of our study we examined the extracts tested for their tyrosinase inhibitory activity. The results show that most of the extracts tested did not influence the activity of tyrosinase *in vitro.* Interestingly, extract P3, almost inactive for melanoma cells, inhibited both mono- and diphenolase activity of tyrosinase in 17.0 ± 2.5 and 30.4 ± 3.6%, respectively, while extract P2 which was highly potent for melanoma cells revealed only diphenolase inhibitory activity of 21.3 ± 2.4%, at the highest concentration tested. However, these effects were few times lower than the reference kojic acid (IC_{50} 0.03 mg/mL). A study of five other *Cladonia* species acetone extracts indicated low inhibition of tyrosinase only for *C. aggregata* (21.5%) and *C. dimorphoclada* (16.5%),[18] which is comparable to our results. However, our study is the first to demonstrate the influence of *Cladonia mitis* extracts on tyrosinase activity.

Conclusions

The results showed that the *C. mitis* acetone extracts revealed a dose-dependent antimelanoma potential and were not toxic to normal skin keratinocytes at lower concentrations. For some of the extracts, anti-tyrosinase activity was also noted. Although the cytotoxic activity of the extracts did not correlate with the content of usnic acid, it is important to emphasize that the susceptibility of melanoma cells to the extract and usnic acid differed substantially. Taking these results into account, it is possible that the topical application of *C. mitis* extracts as adjuvants in cutaneous melanoma could have beneficial effects. Further *in vitro* and *in vivo* safety studies of the tested extracts should also be performed.

Experimental Section

Chemicals and Reagents

The authentic (+)-usnic acid standard and HPLC-grade methanol were purchased from Sigma–Aldrich (Germany). HPLC-grade acetone, and chloroform were purchased from Chempur (Poland). Orthophosphoric acid, toluene, and acetic acid were purchased from Avantor Performance Materials Poland S.A (Poland).

Lichen Material

Cladonia mitis thalli were collected from 19 places along the transect of northern-central Europe, from Norway, through Sweden to Poland, from July 2017 to April 2018. Details of the location of the sample collection have been described previously.[10] The abbreviations of the samples are presented in *Table 1*.

Extraction and Quantitative HPLC Analysis

Cladonia mitis samples were extracted using the previously optimized extraction method.[12] The lichen thalli were air-dried, thoroughly cleaned, and then shredded. Then 0.2 g of each sample was heat-reflux extracted with 20 ml of acetone for 1 h. The extracts were transferred to 10 ml volumetric flasks, filtered using 0.45 μm membrane filters and then analyzed using high performance liquid chromatography. Each sample was extracted in three replicates. Quantitative analysis of usnic acid was performed on a Dionex HPLC system, as previously described,[10] and the results are presented in *Table 1*.

Usnic Acid Isolation

5.0 g of lichen material (sample S11) was weighed and extracted under the conditions mentioned above with 300 ml of acetone. Usnic acid was isolated by preparative thin-layer chromatography, as previously described.[19] The identity of the isolated substance was established by comparison with the standard usnic acid by qualitative HPLC analysis.

Cell Lines and Culture Conditions

Human cancer and the corresponding normal cell lines, used in the study, were: HTB140 – melanoma cells derived from the metastatic site (lymph node), ATCC Hs 294T; A375 – malignant melanoma, ATCC CRL-1619; WM793 – stage I primary melanoma, RRID: CVCL 8787; HaCaT – skin keratinocytes. Cells were grown under standard conditions of 37 °C, 5% CO2, relative humidity and culture medium (DMEM/F12 for WM 793; DMEM High Glucose for HTB140, A375, HaCaT), supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (10 000 U penicillin and 10 mg streptomycin/mL). All supplements and culture media were from Sigma–Aldrich.

Viability Assay

Before the experiment, cells were seeded in 96-well plates for 24 h (1.5×10^4 cells/well). Culture medium was replaced with a fresh medium containing different concentrations of the extracts tested (10–100 μg/mL, in DMSO) and incubated for 24 h. After that time, the viability of the cells was examined by MTT assay, as described previously.[20] Doxorubicin was used as a standard. Each experiment was carried out in triplicate. The absorbance was measured at 570 nm using a Biotek Synergy micro-plate reader (BioTek Instruments Inc., Winooski, VT, USA). Cell viability was expressed as percent of living cells.

Anti-Tyrosinase Assay

The ability of the tested extracts to inhibit tyrosinase activity was evaluated as previously described.[21] The tests were performed in 96-well plates. Both monophenolase and diphenolase tyrosinase activity was examined, using L-tyrosine or L-DOPA as substrates, respectively. Kojic acid was used as a standard. All analyzes were performed in triplicate. The absorbance was measured at 492 nm using a microplate reader. Tyrosinase inhibition was expressed in %.

Statistical Analysis

A single-factor analysis was performed using the ANOVA test in the STATISTICA v.13 program (Stat-soft, Tulsa, OK, USA). The following significance levels were used: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Values < 0.05 were considered statistically significant.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

Conceptualization A.G., P.P., M.G.; Methodology A.G., M.G., D.W.-B.; Formal analysis M.G., D.W.-B.; Original-draft preparation A.G., M.G., P.P., I.P., D.W.-B.; Visualization P.P., M.G.; Funding acquisition A.G.; Supervision A.G., I.P. All authors have read and agreed with this version of the manuscript.

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