Identification and Quantitation of Usnic Acid from the Lichen *Usnea* Species of Anatolia and Antimicrobial Activity

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Six species of lichens, such as *Usnea florida, Usnea barbata, Usnea longissima, Usnea rigida, Usnea hirta* and *Usnea subflorida,* were collected from different areas of Anatolia (district of Antalya, Karabük, Çankırı, Giresun and Trabzon) in Turkey. Their usnic acid amounts in acetone extracts were determined by HPLC. In addition, antimicrobial activities of these extracts were determined against *Escherichia coli* (ATCC 35218), *Enterococcus faecalis* (RSKK 508), *Proteus mirabilis* (Pasteur Ens. 235), *Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus megaterium.* It was shown that with increasing amount of usnic acid, the antimicrobial activity increased. Usnic acid contents of *Usnea* species varied between 0.22–6.49% of dry weight.

Key words: Usnea, HPLC, Antimicrobial Activity

Introduction

The 'beard-like' lichenized ascomycete genus *Usnea* Hill with its fruticose species sharing a shrubby to pendant thallus, pale, yellowish green branches with radical symmetry, a cartilaginous central axis and usnic acid in the cortex is a beloved genus for beginners in lichenology: just stretch the branch and here comes the axis; this making *Usnea* one of the easiest lichen genera to identify (Clerc, 1998).

Some species of the genus *Usnea* have been used for human benefit throughout history. For this reason, they were identified and defined by taxonomists at the very early ages of human history. Since its first isolation in 1844, usnic acid [2,6-diacetyl-7,9-dihydroxy-8,9*b*-dimethyl-1,3(2*H*9*bH*)-dibenzo-furandione] has become the most extensively studied lichen metabolite and one of the few that is commercially available (Knop, 1844). Usnic acid is uniquely found in lichens, and is especially abundant in genera such as *Alectoria, Cladonia, Usnea, Lecanora, Ramalina* and *Evernia*. Many lichens and extracts containing usnic acid have been utilized for medicinal, perfumery, cosmetic as well as ecological applications. Usnic acid as a pure substance has been formulated in creams, toothpaste, mouthwash, deodorants and sunscreen products, in some cases as an active principle, in others as a preservative. In addition to the antimicrobial activity against human and plant pathogens, usnic acid has been shown to exhibit antiviral, antiprotozoal, antiproliferative, antiinflammatory and analgesic activities. Ecological effects, such as antigrowth, antiherbivore and anti-insect properties, have also been demonstrated (Ingolfsdottir, 2002). Additionally, *Usnea* species have been used in Asia, Africa and Europe for pain relief and fever control (Okuyama *et al.,* 1995). *U. barbata* was allegedly used by Hippokrates to treat urinary complaints and *U. longissima* ('Sun-Lo') by the Chinese in wound healing and as an expectorant (Shibata *et al.,* 1948). Extracts of *U. barbata* have been used as a source of usnic acid in modern-day cosmetic and pharmaceutical preparations. In Argentina *U. densirostra*, known as 'Barba del la Piedra', is sold for various disorders (Correche *et al.,* 1998; Ingolfsdottir, 2002).

The examined species were collected from different areas of Anatolia (Turkey) and we report on the antibacterial activities of acetone extracts of *Usnea florida, Usnea barbata, Usnea longissima, Usnea rigida, Usnea hirta* and *Usnea subflorida.* Additionally the antimicrobially active compound usnic acid in acetone extracts was quantified by

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HPLC. For usnic acid isolation from lichen material a previously improved protocol for HPLC studies was used (Cansaran *et al.,* 2006). This is the first study with HPLC technics on the *Usnea* genus and it focused on revealing the antimicrobial activity and also defining usnic acid quantity of six species used in the study.

Materials and Methods

Lichen material

The samples were dried at room temperature and foreign matter was removed prior to grinding. The lichen samples are stored in the herbarium of Ankara University ANK (Ankara University, Department of Botany, Ankara, Turkey). The collection localities are as follows; *Usnea subflorida,* Antalya Termosos Natural Park Güllük Mountain, 830 m; *Usnea florida,* Karabük-Yenice Yaylacık Forest Elmaören Locality, 45° 38′ E, 45° 43′ N, 780 m; *Usnea barbata*, Trabzon-Uzungöl-Soğanlı Locality, 37° 61′ E, 44° 94′ N, 1799 m; *Usnea longissima,* Giresun-Kümbet Area Centre, 1710 m; *Usnea hirta,* Çankırı Yapraklı, Popirunkaşı Hill, 40° 47′ E, 33° 46′ N, 1750 m; *Usnea rigida,* Giresun Dereli-Kulakkaya Area, 38° 20′ E, 40° 45′ N, 1546 m.

Determination of antimicrobial activity

The test microorganisms Escherichia coli (ATCC 35218), Enterococcus faecalis (RSKK 508), Proteus mirabilis (Pasteur Ens. 235), Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Pseudomonas aeruginosa were obtained from Refik Saydam National Type Culture Collection (RSSK) and Ankara University, Faculty of Science, Department of Biology.

Usnic acid was isolated from lichen material according to a protocol given by Cansaran *et al.* (2006). The extraction protocol was as follows: From dried lichen samples 0.05 g were weighed and put into screw capped glass tubes. Extraction was performed by adding 10 ml of acetone and 1 h incubation at room temperature. Chemicals used for extraction were obtained from Sigma and were of the highest grade available. At the end of the incubation period tubes were centrifuged to remove lichens from supernatants. These extracts were used in the experiments.

For screening of antimicrobial activity the agar disc diffusion method was used. The extracts (50 μl) were dried on 6 mm filter paper discs. In addition control discs were prepared with solvents free of lichen extract in order to determine the antimicrobial activity of the solvent acetone. Tetracycline (30 μg/disc) was used as reference. For antimicrobial assays, all bacterial strains were grown in nutrient broth medium (Oxoid) for 24 h at 37 °C. Then 0.1 ml of each culture of bacteria was spread on nutrient agar plate surfaces. After that, discs were placed onto agar Petri plates and incubated. The inhibitory activity was indicated by clear zones around the discs and inhibition zone diameters were measured in mm after incubation for 24 h at 37 °C (Perry *et al.,* 1999). All tests were performed in triplicate.

HPLC analysis of the lichen samples

HPLC analyses were performed as indicated previously (Cansaran *et al.*, 2006). Air-dried lichens were ground and extracted in 0.05 g amount of 10 ml acetone at room temperature (20–22 °C). The extracts were taken to darkness and stored at 4 °C until HPLC analysis. Before analysis extracts were passed through 0.45 μm filters and then injected into the HPLC system in amounts of 20 μl.

All of the chemicals used in the experiments were of HPLC grade from Sigma. A stock solution of 1 mg/ml usnic acid was prepared in acetone. An appropriate dilution of this stock solution was made with acetone. All of the standards were placed in an autosampler and analyzed. Calibration curves for usnic acid were obtained with seven samples of various concentrations using linear regression analysis (Fig. 1).

A Thermo Finnigan HPLC System equipped with a Surveyor LC pump, Surveyor photodiode array detector, Surveyor autosampler and data processor (ChromQuest 4.01) was used. Reverse phase Shim-pack CLC-ODS (M) (5 μm particle

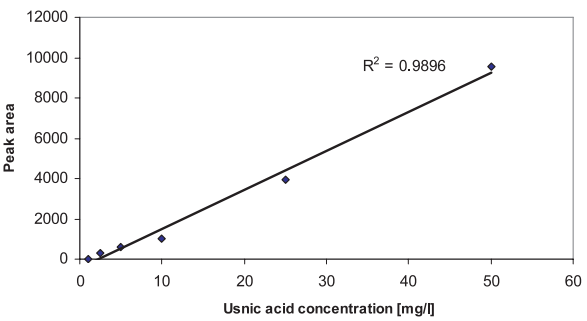

Fig. 1. Calibration curve of usnic acid (Sigma).

size, 250 mm × 4.6 mm I.D.) stainless steel column was used. Flow rate was 0.8 ml/min. For usnic acid detection at 245 nm, a mixture of methanol and phosphate buffer (pH 7.4) (70:30 v/v) was used as mobile phase. 20 μl aliquots of the extracts were injected into the HPLC system. Each analysis was carried out in triplicate.

Results

In this study we tested the antimicrobial activity of the acetone extract of *Usnea florida, U. barbata, U. longissima, U. rigida, U. hirta* and *U. subflorida* against seven test bacteria. The study indicated that lichen extracts have antimicrobial effects against the tested bacteria at different rates. Results from antimicrobial activity tests are given in Table I.

The acetone extracts of *Usnea subflorida, U. florida* and *U. barbata* were found to be effective on most of the tested bacteria. The extract of *Usnea subflorida,* which is the most efficient, showed the highest inhibition effect on *B. subtilis* and *B. megaterium.* This extract also inhibited the growth of Gram-negative bacteria such as *E. coli* and *P. mirabilis.* When the inhibition zones obtained from *Usnea subflorida* were compared with that of a standard antibiotic, it was determined that *E. coli* and *P. mirabilis* were more susceptible to the lichen extract. All the bacteria were found to be less susceptible to the acetone extracts obtained from other *Usnea* species compared to *Usnea subflorida. B. subtilis* and *B. megaterium* seemed to be susceptible to the acetone extracts of all tested *Usnea* species.

Additionally, we determined the antimicrobially active substance usnic acid quantitatively in acetone extracts of *U. florida, U. barbata, U. longissima, U. rigida, U. hirta* and *U. subflorida* by HPLC. Identification of peaks in the chromatograms of lichen extracts was accomplished by comparison of their retention times with that of standard usnic acid. Usnic acid amounts and retention times in the acetone extracts of *U. florida, U. barbata, U. longissima, U. rigida, U. hirta* and *U. subflorida* are given in Table II. The most interesting result is the highest amount of usnic acid with about 6.49% of dry lichen weight in *Usnea subflorida* sample collected from Antalya province.

Table II. Usnic acid content and retention times of lichen species.

Species	% of usnic acid in dry weight	Retention time [min]
Usnea subflorida	6.49 ± 0.01	11.4
Usnea florida	2.36 ± 0.37	13.9
Usnea barbata	2.16 ± 0.67	13.8
Usnea longissima	1.12 ± 0.11	12.2
Usnea hirta	0.68 ± 0.04	13.1
Usnea rigida	0.22 ± 0.01	11.5

Discussion

Many papers deal with the biological activities of isolated usnic acid, especially for pharmaceutical purposes, or in relation to taxonomic problems, while limited information is available about identification and quantitation of usnic acid from *Usnea* species. In this research, the antimicrobial activity of acetone extracts of lichens was tested against different Gram-positive cocci, bacilli and Gram-negative bacilli. From our results, it could be concluded that Gram-positive bacilli are inhibited effectively.

Table I. Antimicrobial activity of various lichen extracts.

	Mean (average) inhibition zone [mm][a]						
	Usnea subflorida	*Usnea florida*	*Usnea barbata*	*Usnea longissima*	*Usnea hirta*	*Usnea rigida*	Tet[b]
Escherichia coli (ATCC 35218)	18 ± 0.01	–	–	–	–	–	12
Enterococcus faecalis (RSKK 508)	–	–	–	–	–	–	30
Proteus mirabilis (Pasteur Ens. 235)	20 ± 0.01	–	–	–	–	–	8
Staphylococcus aureus	–	–	–	–	–	–	40
Bacillus subtilis	30 ± 0.01	21 ± 0.01	20 ± 0.01	15 ± 0.02	14 ± 0.01	12 ± 0.01	26
Bacillus megaterium	31 ± 0.01	22 ± 0.01	22 ± 0.02	17 ± 0.02	11 ± 0.01	10 ± 0.01	20
Pseudomonas aeruginosa	–	–	–	–	–	–	20

[a] Includes diameter of disc (6 mm). [b] Tet, tetracycline. (–), no inhibition.

Usnic acid is extensively distributed in species of *Cladonia, Usnea, Lecanora, Ramalina, Evernia, Parmelia* and other lichen genera. *Alectoria* species are often rich sources of usnic acid, and yields of up to 6% have been reported (Proska *et al.,* 1996). This study showed that usnic acid produced in large amounts in *Usnea subflorida* made up 6.49% of the dry lichen weight.

Usnea barbata extracts showed significant activity against the Gram-positive bacteria with minimum inhibitory concentrations as low as 0.1 mg/ml on *Bacillus subtilis, Enterococcus faecalis, Micrococcus viradans* and *Staphylococcus aureus.* The acetone extract was the most active while the water extract showed the least activity against the microbes (Madamombe and Afolayan, 2003). In addition Behera *et al.* (2005) determined the antimicrobial and antioxidant activities of *Usnea ghattensis* and found that *U. ghattensis* was active against *S. aureus, B. licheniformis, B. subtilis* and *B. megaterium* (Behera *et al.,* 2005).

In a cancer chemoprevention assay designed to detect potential inhibitors of tumour promotion, (+)-usnic acid isolated from *U. longissima* showed potent inhibitory effects (ED_{50} 1.0 μg/ml) against Epstein-Barr virus activation induced by teleocidin B-4, a potent tumour promoter (Yamamoto *et al.* 1995). Commercially obtained (–)-usnic acid was less active (ED_{50} 5.0 μg/ml).

The presence of usnic acid in the lichen *Usnea laevis* Nyl. from the Venezuelan Andes was detected by chromatographic (TLC) and spectroscopic (IR, MS, ^{1}H NMR) methods. This compound was present in a content of 2.7% in the thallus. Usnic acid has a reported antibiotic activity and the lichen is utilized for medicinal purposes by Andean farmers (Marcano *et al.,* 1999).

According to the literature the high antimicrobial activity of usnic acid has long been known and our results show a similar finding for the antimicrobial activity of usnic acid. The maximum antibacterial efficiency among six *Usnea* species was exhibited by *Usnea subflorida,* which has the highest usnic acid level (6.49% of dry lichen weight).

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Behera B. C., Verma N., Sonone A., and Makhija U. (2005), Antioxidant and antibacterial activities of lichen *Usnea ghattensis in vitro.* Biotechnol. Lett. **27**, 991–995.

Cansaran D., Çetin D., Halici G., and Atakol O. (2006), Determination of usnic acid in some *Rhizoplaca s*pecies from Middle Anatolia and their antimicrobial activities. Z. Naturforsch. **61c**, 47–51.

Clerc P. (1998), Species concepts in the genus *Usnea* (lichenized Ascomycetes). Lichenologist **30**, 321–340.

Correche E. R., Carrasco M., Escudero M. E., Velazquez L., de Guzman A. M. S., Giannini F., Enriz R. D., Jauregui E. A., Cenal J. P., and Giordano O. S. (1998), Study of the cytotoxic and antimicrobial activities of usnic acid and derivatives. Fitoterapia **69**, 493–501.

Ingolfsdottir K. (2002), Usnic acid. Phytochemistry **61**, 729–736.

Knop W. (1844), Chemisch-physiologische Untersuchung über die Flechten. Ann. Chem. Pharm. **49**, 103–124.

Madamombe I. T. and Afolayan A. J. (2003), Evaluation of antimicrobial activity of extracts from South African *Usnea barbata.* Pharmaceut. Biol. **41**, 199–202.

Marcano V., Alcocer V. R., and Mendez A. M. (1999), Occurrence of usnic acid in *Usnea laevis* Nylander (lichenized Ascomycetes) from the Venezuelan Andes. J. Ethnopharmacol. **66**, 343–346.

Okuyama E., Umeyama K., Yamazaki M., Kinoshita Y., and Yamamoto Y. (1995), Usnic acid and diffractaic acid as analgesic and antipyretic components of *Usnea diffracta.* Planta Med. **61**, 113–115.

Perry N. B., Benn M. H., Brennan N. J., Burgess E. J., Ellis G., Galloway D. J., Lorimer S. D., and Tangney R. S. (1999), Antimicrobial, antiviral and cytotoxic activity of New Zealand lichens. Lichenologist **31**, 627–636.

Proska B., Sturdikova M., Pronayova N., and Liptaj T. (1996), (–)-Usnic acid and its derivatives. Their inhibition of fungal growth and enzyme activity. Pharmazie **51**, 195–196.

Shibata S., Ukita T., Tamurta T., and Miura Y. (1948), Relation between chemical constitutions and antibacterial effects of usnic acid and derivatives. Jpn. Med. J. **1**, 152–155.

Yamamoto Y., Miura Y., Kinoshita Y., Higuchi M., Yamada Y., Murakami A., Ohigashi H., and Koshimizu K. (1995), Screening of tissue cultures and thalli of lichens and some of their active constituents for inhibition of tumor promoter-induced Epstein-Barr virus activation. Chem. Pharm. Bull. **43**, 1388–1390.