Determination of Usnic Acid in Some *Rhizoplaca* Species from Middle Anatolia and their Antimicrobial Activities

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Three species of lichens, *Rhizoplaca chrysoleuca* (Sm.) Zopf, *Rhizoplaca melanopthalma* (DC.) Leuckert & Poelt and *Rhizoplaca peltata* Ramonds Leuckert, were collected from middle Anatolia, Erciyes Mountain (Kayseri) in Turkey. Their usnic acid amounts were determined by HPLC in acetone extracts. 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, Bacillus megaterium* and *Pseudomonas aeruginosa.* It was shown that, as the usnic acid amount increased, the antimicrobial activities increased too. The usnic acid contents of *Rhizoplaca* species varied between 0.19–4.0% dry weight.

Key words: Rhizoplaca, Lichen, Antimicrobial Activity

Introduction

The genus *Rhizoplaca* Zopf belongs to the *Lecanorales.* It includes crustose lichens with *Trebouxia* as photobiont, which are most frequently yellowish green to yellow-gray, but sometimes gray, and foliose, umbilicate lichens, rarely crustose or fruticose. Thalli are usually rounded and often lumpy; they are 10–30 mm in diameter and the lower surface is pallet to black, without rhizines. The photobiont is green. Apothecia are lecanorine, large and orange-pink to dark green in color or almost black. Spores are colorless; they are 1-celled, ellipsoid and small. The cortex gives a positive result with yellow orange color (usnic acid) or a rarely negative result (lacking usnic acid) with KC (K, 10% KOH; C, commercial bleach) reagent. The medulla often contains placodiolic or pseudoplacodiolic acid and other depsides, depsidones, and triterpenes in various combinations. It is located on siliceous or calcareous rocks, or unattached on soil, in open, especially dry sites (Brodo *et al.*, 2001). While the lower surface is rough, broken into areoles with white cracks, apothecial disks are yellowish brown, and medulla are Pd + orange (pannarin) in *R. peltata.* In *R. melanopthalma* the lower surface is smooth; apothecial disks are yellowish brown to greenish or black, pruinose; medulla are Pd + (with psoromic acid) bright yellow, or less frequently Pd – (without psoromic acid).

The study of lichens and lichen substances, from the antibiotic point of view, started in 1944, when Burkholder and Evans (1944) published the first qualitative study of the antibiotic properties of lichens (Ahmadjian and Hale, 1973). Although several detailed studies on the lichen from Turkey provinces and its antimicrobial activity have recently been published (Dülger *et al.*, 1997; Kırmızıgül *et al.*, 2003), knowledge of the lichen and its antimicrobial activity is still lacking.

Of the more than 20,000 known species of lichens, only a few have been analyzed and identified as containing biologically active secondary compounds. A prominent example is the antimicrobial compound usnic acid [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2*H*9β*H*)-dibenzofurandione], commonly found in the genus *Usnea* (Elix, 1996) (Fig. 1). Usnic acid is a yellowish pigment produced by several lichen species. It has been documented to have antihistamine, spasmolytic, antiviral, and antibacterial activities. Two

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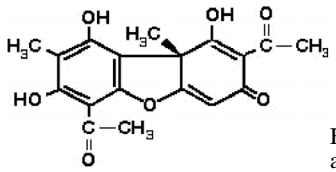

Fig. 1. Structure of usnic acid.

biologically active natural enantiomers of usnic acid, differing in the orientation of the methyl group at 9b on the otherwise rigid molecule, have been identified as showing different biological activities and mechanisms of action. Broksa *et al.* (1996) reported that (–)-usnic acid inhibited urease and arginase activity. There are several reports (Lauterwein *et al.*, 1995; Ghione *et al.*, 1988) that the (+)-enantiomer is a more effective antimicrobial agent, and usnic acid specifically inhibits *p*-hydroxyphenylpyruvate dioxygenase (Romagni *et al.*, 2000) and is used as a preservative in cosmetic creams (Seifert and Bertram, 1995).

HPLC is an ideal tool for detecting trace substances, analyzing small samples, quantifying phenolic lichen metabolites, and providing structural information from retention characteristics. A standardized method for elution HPLC was developed by Feige *et al.* (1993) using an UV detector. Yoshimura *et al.* (1994) described the use of a photodiode array detector for HPLC analysis of lichen substances (Kranner *et al.*, 2002).

In this study, the antimicrobial activities of acetone extracts of *Rhizoplaca chrysoleuca*, *Rhizoplaca melanopthalma* and *Rhizoplaca peltata*, which are three identified *Rhizoplaca* species in Turkey, were tested against different Gram-positive cocci, bacilli and Gram- negative bacilli. The antimicrobially active compound usnic acid in acetone extracts was quantified by HPLC.

Experimental

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 Erciyes University (Erciyes University, Department of Botany, Kayseri, Turkey). The collection localities are as follows; *Rhizoplaca melanopthalma*, Turkey, (Prov.) Kayseri (38) Erciyes Mountain; western slope of Erciyes Mountain (along the telepherics), 38°32′N, 35°30′E, 2500–2600 m (leg. & det. M. G. Halıcı); *Rhizoplaca chrysoleuca*, Turkey, (Prov.) Kayseri (38) Erciyes Mountain; western slope of Koç Mountain, 38°32′N, 35°32′E, 2200–2300 m (leg. & det. M. G. Halıcı); *Rhizoplaca peltata*, Turkey, (Prov.) Kayseri (38) Erciyes Mountain; north of Perikartın (northern slope of Erciyes Mountain), 38°35′N, 35°27′E, 2300 m (leg. & det. M. G. Halıcı).

Determination of antimicrobial activity

Test microorganisms

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

Preparation of lichen extracts for antimicrobial activity assays

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 with 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 incubation period tubes were centrifuged to remove lichens from supernatants. These extracts were used in the experiments.

Antimicrobial activity assays

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.

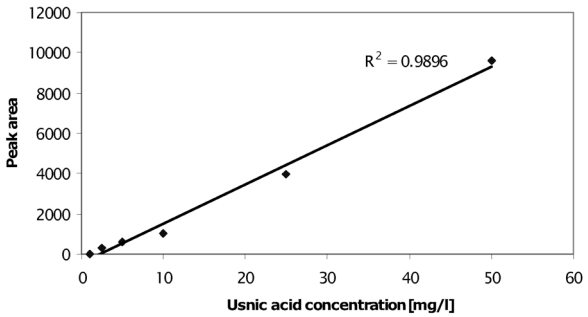

Fig. 2. Calibration curve of usnic acid (Sigma).

HPLC analysis of the lichen samples

Sample preparation for HPLC analysis

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.

Standard and solvents

All of the chemicals used in the experiments were HPLC grade from Sigma *i.e.* of highest purity. 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. 2).

Analytical conditions and apparatus

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 size, in a 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 methanol/phosphate buffer (pH 7.4) (70:30 v/v) was used as the 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 *Rhizoplaca chrysoleuca*, *Rhizoplaca melanopthalma* and *Rhizoplaca peltata* against seven test bacteria. The study showed 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 extract of *Rhizoplaca chrysoleuca* was found to be very effective on all the bacteria (except for *P. aeruginosa*). *R. chrysoleuca* showed the highest inhibition effect on *B. megaterium* and *B. subtilis*. This extract also considerably inhibited the growth of Gram-negative bacteria such as *E. coli* and *P. mirabilis*. When the inhibition zones obtained from *R. chrysoleuca* were compared with that of a standard antibiotic, it was determined that *B. megaterium*, *E. coli* and *P. mirabilis* were more susceptible to the lichen extract. All the bacteria were found to be less susceptible to the ace-

Table I. Antimicrobial activity of various lichen extracts.

	Mean (average) inhibition zone [mm]*			
	Rhizoplaca chrysoleuca	*Rhizoplaca peltata*	*Rhizoplaca melanopthalma*	Tet
Escherichia coli (ATCC 35218)	25	13	10	12
Enterococcus faecalis (RSKK 508)	9	7	–	30
Proteus mirabilis (Pasteur Ens. 235)	20	15	–	8
Staphylococcus aureus	15	–	–	40
Bacillus subtilis	26	20	9	26
Bacillus megaterium	33	30	15	20
Pseudomonas aeruginosa	–	–	–	20

* Includes diameter of disc (6 mm).
Tet, tetracycline; (–), no inhibition.

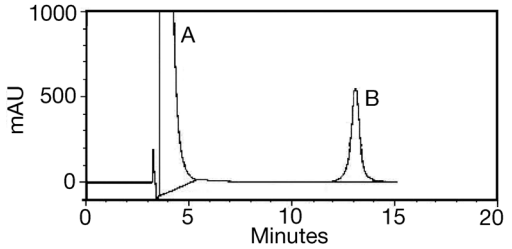

Fig. 3. Analysis of usnic acid from *Rhizoplaca* by HPLC: A, solvent (t_R = 3.9 min); B, usnic acid (t_R = 13.1 min).

tone extracts obtained from *R. peltata* and *R. melanopthalma* than from *R. chrysoleuca.* Similar to *R. chrysoleuca*, *R. peltata* and *R. melanopthalma* showed the highest inhibition effect on *B. megaterium.*

After that, we continued to determine the antimicrobially active substance usnic acid in an acetone extract of *Rhizoplaca* genus. Quantitative analysis of usnic acid in *Rhizoplaca chrysoleuca*, *Rhizoplaca melanopthalma* and *Rhizoplaca peltata* was achieved using HPLC. The identification of peaks in chromatograms of lichen extracts was accomplished by comparison of retention times with that of standard usnic acid. A sample of representative chromatograms is shown in Fig. 3. Usnic acid amounts and retention times in the acetone extracts of *Rhizoplaca chrysoleuca*, *Rhizoplaca melanopthalma* and *Rhizoplaca peltata* are given in Table II. The highest amount of usnic acid was found to be about 4% of the dry lichen weight in *Rhizoplaca chrysoleuca.*

Discussion

In this study, the antimicrobial activity of acetone extracts of lichens was tested against different Gram-positive cocci and bacilli and Gram-negative bacilli. From our results, it could be concluded that Gram-positive bacilli are inhibited effectively in general.

In recent years a number of studies have focused on usnic acid. Usnic acid is a secondary metabolite which has antimicrobial activity.

According to Tay *et al.* (2004) the acetone extract of the lichen *Ramalina farinacea* showed antimicrobial activity against *B. subtilis*, *Listeria monocytogenes*, *P. vulgaris*, *S. aureus*, *S. faecalis*, *Yersinia enterocolitica*, *Candida albicans* and *C. glabrata.* They demonstrated that the (+)-usnic acid constituent of *Ramalina farinacea* is the major antimicrobial agent in this lichen.

The *in vitro* susceptibility of pathogenic Gram-positive bacteria, anaerobic bacteria, mycobacteria and some fungi towards (+)- and (−)-usnic acids has been confirmed by Ingolfsdottir (2002) and Lauterwein *et al.* (1995). Lauterwein *et al.* (1995) determined *in vitro* activities of (+)- usnic acid, (−)-usnic acid, and vulpinic acid against aerobic and anaerobic microorganisms. They found that these lichen compounds did not inhibit Gram-negative rods or fungi at concentrations lower than 32 μg/ml but were active against clinical isolates of *E. faecalis, E. faecium*, and *S. aureus.* Also it was reported that both forms of usnic acid inhibited the growth of *Mycobacterium tuberculosis* and *M. tufu in vitro* at a relatively low concentration (Krishna and Venkataramana, 1992). The antibacterial activity of usnic acid against *Streptococcus mutans* has been examined by Ghione *et al.* (1988). As it can be seen from literature the high antimicrobial activity of usnic acid has long been known and our results show similar findings for the antimicrobial activity of usnic acid. The maximum antibacterial efficiency among the three *Rhizoplaca* species was exhibited by *Rhizoplaca chrysoleuca*, which has the highest usnic acid level.

Usnic acid is extensively distributed in species of *Cladonia*, *Usnea*, *Leconora*, *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 (Broksa *et al.*, 1996). This study showed that usnic acid produced

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

Species	% of usnic acid of dry weight	Retention time [min]
Rhizoplaca chrysoleuca	4 ± 0.07	13.1
Rhizoplaca melanopthalma	0.19 ± 0.01	13.2
Rhizoplaca peltata	0.53 ± 0.04	12.1

in large amounts in *Rhizoplaca chrysoleuca* made up 4% of the dry lichen weight. Although the literature contains many studies, this is the first report about the usnic acid content of *Rhizoplaca* sp. in Turkey.

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