

Evaluation of the Antimicrobial Activity of the Acetone Extract of the Lichen *Ramalina farinacea* and its (+)-Usnic Acid, Norstictic Acid, and Protocetraric Acid Constituents

Turgay Tay^a, Ayşen Özdemir Türk^{b,*}, Meral Yılmaz^b, Hayrettin Türk^a, and Merih Kivanç^b

^a Anadolu University, Department of Chemistry, 26470 Eskişehir, Turkey

^b Anadolu University, Department of Biology, 26470 Eskişehir, Turkey.

Fax: +902223204910. E-mail: aturk@anadolu.edu.tr

* Author for correspondence and reprint requests

Z. Naturforsch. **59c**, 384–388 (2004); received December 18, 2003/January 26, 2004

The acetone extract of the lichen *Ramalina farinacea* and its (+)-usnic acid constituent showed antimicrobial activity against *Bacillus subtilis*, *Listeria monocytogenes*, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Yersinia enterocolitica*, *Candida albicans*, and *Candida glabrata*. Norstictic acid was active against *Aeromonas hydrophila* as well as the above microorganisms except *Yersinia enterocolitica*. Protocetraric acid showed activity only against the tested yeasts *Candida albicans* and *Candida glabrata*. The MIC values of the extract as well as of the three substances were determined. No antifungal activity of the acetone extract has been observed against ten filamentous fungi.

Key words: *Ramalina farinacea*, Antimicrobial Activity, Lichen Compounds

Introduction

Lichens have been used for medical purposes since ancient times and are known to produce unique secondary metabolites, a number of which have considerable biological activities such as antimicrobial, antiherbivore, and antibiotic (Vartia, 1973; Richardson, 1988; Lawrey, 1989; Elix, 1996). Secondary metabolites in lichens are produced by the fungus alone and secreted onto the surface of lichen's hyphae in amorphous forms or crystals. Up to now about 350 secondary metabolites are known from lichens and approximately 200 have been characterized. Papers dealing with the biological activity of lichens and lichen substances started to appear after World War II. Lists of the antibacterial and antifungal activities of lichen compounds and lichens against bacteria and fungi can be found in a review and a book (Huneck, 1999, 2001).

Among the lichen substances, the most widely distributed and the most extensively investigated one, without doubt, is usnic acid (Fig. 1). It occurs in two enantiomeric forms and (+)-usnic acid has *R* configuration in its chiral center. Usnic acid has been used in several countries as an atopic antibiotic for human skin diseases and first reports on its antibacterial activity are about 50 years old. Two recent reviews summarize its antimicrobial,

antiprotozoal, antiviral, antiproliferative, anti-inflammatory, analgesic, antipyretic, and anti-tumour activities as well as some other properties such as UV protection, allergenic potential, toxicity (Cocchietto *et al.*, 2002; Ingólfssdóttir, 2002). Ingólfssdóttir's review presents a comprehensive list for the antimicrobial activity of (+)-usnic acid and (–)-usnic acid against gram positive and gram negative, anaerobic bacteria, mycobacteria, and yeast/fungi with the relevant references. Ghione *et al.* (1988) reported the antibacterial activity of usnic acid against *Streptococcus mutans*, *Streptococcus pyogenes*, and *Staphylococcus aureus*. 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 *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, and some anaerobic bacteria. Both enantiomeric forms of usnic acid inhibited the growth of *Mycobacterium tuberculosis* and *Mycobacterium tufo* *in vitro* at a relatively low concentration (Krishna and Venkataramana, 1992). *In vitro* activities of five common lichen compounds were screened for *Mycobacterium aurum* by Ingólfssdóttir *et al.* (1998). Among

their test compounds, (+)-usnic acid from *Cladonia arbuscula* exhibited the highest activity against *M. aurum* with a MIC value of 32 µg/ml. Perry and coworkers (1999) published the antimicrobial, antiviral and cytotoxic activity results of screening 69 species of New Zealand lichens as well as 6 pure compounds including usnic acid. Usnic acid (60 µg per disk) showed antimicrobial activity against *Bacillus subtilis*, *Candida albicans* and *Trichophyton mentagrophytes*. Furthermore, 30 µg, 7.5 µg, 1.5 µg and 0.4 µg usnic acid containing disks also showed antimicrobial activities against *Bacillus subtilis* but these quantities were not tested against the other two microorganisms.

To the best of our knowledge no information about the antimicrobial activities of norstictic acid and protocetraric acid is available for the last decade in the literature.

Experimental

Microorganisms

Staphylococcus aureus (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Fusarium solani* (ATCC 12820) (from the American Type Culture Collection/USA), *Proteus vulgaris* (NRRL B-123), *Bacillus cereus* (NRRL B-3711), *Bacillus subtilis* (NRRL B-744), *Streptococcus faecalis* (NRRL B-14617), and *Penicillium notatum* (NRRL 807) (from the Northern Regional Research Laboratory of the USDA, Peoria, Illinois/USA), *Listeria monocytogenes* (from Ankara University, Faculty of Agriculture, Ankara, Turkey), *Yersinia enterocolitica* and *Aeromonas hydrophila* (from Ankara University, Faculty of Veterinary, Ankara, Turkey), *Pseudomonas syringae* pv. *tomato* (TPPB 4212), *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium culmorum*, and *Cochliobolus sativus* (from the Agriculture Research Center, Eskişehir/Turkey), *Candida albicans* and *Candida glabrata* (from Osmangazi University, Faculty of Medicine, Eskişehir, Turkey), *Alternaria tenuissima*, *Alternaria alternata*, *Alternaria citri*, and *Penicillium parasiticus* (from Trakya University, Department of Biology, Edirne, Turkey), *Klebsiella pneumoniae* and *Salmonella typhimurium* (available in our department) were used as microorganisms. Bacteria and yeasts were kept on nutrient agar and yeast extract agar plates at 4 °C, respectively. Fungal test cultures were subcultured on potato dextrose agar (PDA) for 5–7 d at 25 °C.

Lichen material

Ramalina farinacea was collected in TEMA Forest, Bozdag, Eskişehir Province, Turkey at 1100 m. A herbarium sample is stored at the Herbarium of Anadolu University in the Department of Biology (ANES).

Determination of MIC values of the acetone extract

The Kirby and Bauer disk diffusion method (National Committee for Clinical Laboratory Standards, 1993) has been used to determine MIC values of the acetone extract of the lichen *Ramalina farinacea* against test bacteria and fungi. For extraction, a 10 g air-dried lichen sample was ground and added to 200 ml of acetone. The mixture was sonicated for 30 min, then left at room temperature overnight, and filtered. After removal of the solvent of the filtrate, 130 mg residue was recovered. A stock solution of the residue was prepared with acetone and then sterilized by membrane filtration. From the sterilized stock solution, certain volumes in 1 ml portions were added to 10 sterilized tubes containing 40 sterilized filter paper disks each. If the volume of stock solution to be added was less than 1 ml, its volume was added up to 1 ml with pure acetone before addition. The solvent of the solutions in tubes was allowed to evaporate; this eventually left the residue of the acetone extract of the lichen on disks without solvent. We obtained an array of disks whose residue contents varied from 0.42 mg per disk to 0.82 µg per disk and the amount of the residue on a disk in the array was twice as much as that of the next disk. Before transferring these disks into the microorganism media, the microorganism media were prepared as follows: 250 µl (10⁸ cells/ml) suspensions of the bacteria and yeasts were inoculated onto nutrient agar plate and onto potato dextrose agar, respectively. Then the disks containing an array of different amounts of extract residue were transferred on each plate. Pure acetone-treated and dried disks were used as a negative control agent and chloramphenicol and ketoconazole were used as positive control substances. The bacterial plates were incubated for 24–48 h at 35–37 °C. All experiments were done twice and the MIC values were determined by checking the inhibition zones formed. For the filamentous fungi, another acetone extract of *R. farinacea*, and subsequently an array of disks have been prepared the same way as described above. 500 µl (10⁸ spores/

ml) suspensions of the filamentous fungi spores were inoculated onto potato dextrose agar. The plates were incubated for 5 d at 20–25 °C.

Bioautographic method using thin layer chromatography

A bioautographic method has been carried out as described in our previous work (Yılmaz *et al.*, 2004). On the TLC plates, we observed three major substances and two of them showed antimicrobial activity.

Isolation and characterization of test compounds

An acetone extract of an air-dried sample of 40 g of *Ramalina farinacea* was obtained as described above and the test compounds, identified later as (+)-usnic acid, norstictic acid, and protocetraric acid, were isolated using a preparative TLC method. The TLC plates (Merck Silica gel 60 F₂₅₄) were developed in solvent system G, which is one of the solvent systems used in the TLC of lichen substances and consists of toluene/ethyl acetate/formic acid (139:83:8 v/v/v). The characterization of the substances was based on comparing *R_f* values in A, C, and G solvent systems with those given in the literature, their melting points and IR spectra (Huneck and Yoshimura, 1996; Culberson *et al.*, 1977; Culberson and Amman, 1979; Orange *et al.*, 2001; Schumm, 2002; Edwards *et al.*, 2003). Furthermore, the TLC of the extract of *Pleurosticta acetabulum*, a control lichen for norstictic acid, was used as reference. A polarimeter was used to determine the enantiomeric form of usnic acid.

Determination of MIC values of (+)-usnic acid, norstictic acid, and protocetraric acid

The MIC values of (+)-usnic acid, norstictic acid, and protocetraric acid were determined using the same procedure described above for the acetone extract. Stock solutions of (+)-usnic acid, norstictic acid, and protocetraric acid in acetone were prepared and subsequently diluted two-fold with acetone for ten times. After the addition of the diluted solutions in 1 ml-aliquots into the sterilized 40 disk-containing tubes and after evaporating the solvent, the amount of the compounds on the disks varied from 12.5 µg to 24 ng for (+)-usnic acid, from 0.38 mg to 0.74 µg for norstictic acid, and from 0.50 mg to 0.97 µg for protocetraric acid.

The determinations of MIC values of (+)-usnic acid, norstictic acid, and protocetraric acid against

the bacteria and the yeasts were carried out as the determination of MIC values of the acetone extract.

Results and Discussion

We tested the antimicrobial activity of the acetone extract of *Ramalina farinacea* against thirteen bacteria, two yeasts, and ten filamentous fungi. The extract showed antimicrobial activity against some of the tested bacteria and the yeasts (Table I) and no activity against the tested filamentous fungi. *B. subtilis*, *L. monocytogenes*, *P. vulgaris*, *S. aureus*, *S. faecalis*, *Y. enterocolitica*, *C. albicans*, and *C. glabrata* were the microorganisms whose growth were inhibited by the extract. Esimone and Adikwu (1999) evaluated the phytochemical constituents, antibacterial, antifungal, and cytotoxic properties of the extracts of *R. farinacea*. The ethyl alcohol, chloroform and *n*-hexane extracts (4 mg per disk) showed antibacterial and antifungal activity against *S. aureus*, *B. subtilis*, *E. coli*, *S. typhimurium*, *P. aeruginosa*, *C. albicans*, *A. niger*, *T. rubrum*, and *T. mentagrophytes*. However, we did not observe any activity against *E. coli*, *P. aeruginosa*, and *S. typhimurium* from our *R. farinacea* extract. The reason for these conflicting results may be due to variations in the experimental conditions. We used disks containing 0.42 mg residue per disk or less in our experiments whereas Esimone and Adikwu used 4 mg residue containing disks in their study. We also determined the MIC values of the extract using the Kirby and Bauer method and the MIC values were remarkably low. We obtained a MIC value of 3.3 µg for seven of the eight microorganisms whose growth were inhibited and 6.6 µg for *B. subtilis*.

The TLC of the acetone extract showed the presence of three major substances in the extract and the bioautographic method proved that two of them are active against some of the microorganisms. We isolated those substances using preparative TLC and identified them as (+)-usnic acid, norstictic acid, and protocetraric acid (Fig. 1). The recovered amounts of (+)-usnic acid, norstictic acid, and protocetraric acid from the extract of 10 g air-dried lichen with 200 ml acetone were 21 mg, 28 mg, and 25 mg, respectively. (+)-Usnic acid and norstictic acid were determined as the active ones in the bioautography. Their activities against thirteen bacteria and two yeasts, the same microorganisms used in the study of the extract,

Table I. MIC values of the acetone extract of *Ramalina farinacea* and its (+)-usnic acid, norstictic acid, and protocetraric acid constituents.

Microorganism	MIC (against 10 ⁷ cells) ^a						
	Acetone extract [$\mu\text{g}/25 \mu\text{l}$]	(+)-Usnic acid [$\mu\text{g}/62.5 \mu\text{l}$]	([mM])	Norstictic acid [$\mu\text{g}/75 \mu\text{l}$]	([mM])	Protocetraric acid [$\mu\text{g}/75 \mu\text{l}$]	([mM])
<i>Aeromonas hydrophila</i>	–	–	–	23.4	(0.84)	–	–
<i>Bacillus cereus</i>	–	–	–	–	–	–	–
<i>Bacillus subtilis</i>	6.6	0.78	(0.036)	93.8	(3.4)	–	–
<i>Escherichia coli</i>	–	–	–	–	–	–	–
<i>Klebsiella pneumoniae</i>	–	–	–	–	–	–	–
<i>Listeria monocytogenes</i>	3.3	0.39	(0.018)	11.7	(0.42)	–	–
<i>Proteus vulgaris</i>	3.3	0.78	(0.036)	46.9	(1.7)	–	–
<i>Pseudomonas aeruginosa</i>	–	–	–	–	–	–	–
<i>Pseudomonas syringae</i>	–	–	–	–	–	–	–
<i>Staphylococcus aureus</i>	3.3	3.1	(0.15)	188	(6.7)	–	–
<i>Streptococcus faecalis</i>	3.3	1.6	(0.073)	23.4	(0.84)	–	–
<i>Salmonella typhimurium</i>	–	–	–	–	–	–	–
<i>Yersinia enterocolitica</i>	3.3	0.39	(0.018)	–	–	–	–
<i>Candida albicans</i>	3.3	0.05	(0.002)	2.9	(0.10)	3.9	(0.14)
<i>Candida glabrata</i>	3.3	0.05	(0.002)	2.9	(0.10)	3.9	(0.14)

^a –: Inactive.

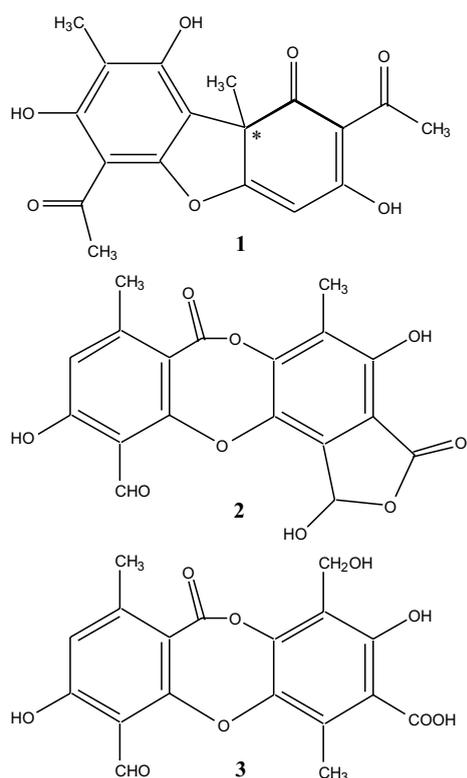


Fig. 1. Chemical structures of usnic acid (1), norstictic acid (2), and protocetraric acid (3).

were tested and their MIC values are given in Table I.

(+)-Usnic acid showed antimicrobial activity against the same microorganisms as the acetone extract did. Among the three compounds it was the most active one having quite low MIC values varying from 0.05 $\mu\text{g}/62.5 \mu\text{l}$ to 3.1 $\mu\text{g}/62.5 \mu\text{l}$. Comparing the MIC values of the extract and (+)-usnic acid, it appears that (+)-usnic acid is the major antimicrobial agent in *Ramalina farinacea*. The high antimicrobial activity of usnic acid has been known for a long time. Lauterwein *et al.* (1995) reported a MIC value range from 2 to 16 $\mu\text{g}/\text{ml}$ for (+)-usnic acid against clinical isolates of *S. aureus*. They also found that (+)-usnic acid along with (–)-usnic acid and vulpinic acid was inactive against *P. aeruginosa*, *E. coli*, and *C. albicans*. Perry and coworkers (1999) observed the antimicrobial activity of usnic acid against *B. subtilis* and *C. albicans*. In one of our studies, we have assayed the antimicrobial activity of the other enantiomeric form namely (–)-usnic acid, obtained from *Cladonia foliacea* against the same bacteria and yeasts (Yılmaz *et al.*, 2004). Although the MIC values of both enantiomers were comparable in our studies, those of (–)-usnic acid were generally lower. Furthermore, (+)-usnic acid did not show any activity against *A. hydrophila* and *B. cereus* whereas (–)-usnic acid did. On the other hand,

(+)-usnic acid was active against *Y. enterocolitica* whereas (-)-usnic acid was not active. Ghione *et al.* (1988) and Lauterwein *et al.* (1995) reported lower MIC values for (+)-usnic acid for some bacteria as well. As Lauterwein *et al.* have found, we did not observe any activity of (+)-usnic acid against *E. coli* and *P. aeruginosa*.

To our knowledge, this study is the first one which reports the antimicrobial activities of norstictic acid and protocetraric acid. Although the MIC values of norstictic acid were much higher

than those of (+)-usnic acid, it was active against *A. hydrophila* and the microorganisms against which (+)-usnic acid was active, except *Y. enterocolitica*. Because of the high MIC value of norstictic acid against *A. hydrophila*, we assume that its amount was not enough or below the MIC value in the acetone extract to inhibit *A. hydrophila* growth. Protocetraric acid was not active against all bacteria tested but active against the yeasts *Candida albicans* and *Candida glabrata* with MIC values as low as 3.9 $\mu\text{g}/75 \mu\text{l}$.

- Cocchietto M., Skert N., Nimis P. L., and Sava G. (2002), A review on usnic acid, an interesting natural compound. *Naturwissenschaften* **89**, 137–146.
- Culberson C. F. and Amman K. (1979), Standardmethode zur Dünnschichtchromatographie von Flechtensubstanzen. *Herzogia* **5**, 1–24.
- Culberson C. F., Culberson W. L., and Johnson A. (1977), Second Supplement to Chemical and Botanical Guide to Lichen Products. The American Bryological and Lichenological Society, St. Louis, Missouri.
- Edwards H. G. M., Newton E. M., and Wynn-Williams D. D. (2003), Molecular structural studies of lichen substances II: atranorin, gyrophoric acid, fumarprotocetraric acid, rhizocarpic acid, calycin, pulvinic acid dilactone and usnic acid. *J. Mol. Struct.* **651–653**, 27–37.
- Elix J. A. (1996), Biochemistry and secondary metabolites. In: *Lichen Biology* (Nash III T. H., ed.). Cambridge University Press, Cambridge, pp. 154–181.
- Esimone C. O. and Adikwu M. U. (1999), Antimicrobial activity and cytotoxicity of *Ramalina farinacea*. *Fitoterapia* **70**, 428–431.
- Ghione M., Parrello D., and Grasso L. (1988), Usnic acid revisited, its activity on oral flora. *Chemoterapia* **7**, 302–305.
- Huneck S. (1999), The significance of lichens and their metabolites. *Naturwissenschaften* **86**, 559–570.
- Huneck S. (2001), New results on the chemistry of lichen substances. In: *Progress in the Chemistry of Organic Natural Products*, 81 (Herz W., Falk H., Kirby G. W., and Moore R. E., eds.). Springer, Wien, New York, pp. 224–230.
- Huneck S. and Yoshimura I. (1996), Identification of Lichen Substances. Springer, Berlin.
- Ingólfssdóttir K. (2002), Usnic acid. *Phytochemistry* **61**, 729–736.
- Ingólfssdóttir K., Chung G. A. C., Vilhjálmur G. S., Gissurarson S. F., and Vilhelmsdóttir M. (1998), Antimycobacterial activity of lichen metabolites *in vitro*. *Eur. J. Pharmaceut. Sci.* **6**, 141–144.
- Krishna D. R. and Venkataramana D. (1992), Pharmacokinetics of D-(+)-usnic acid after intravenous and oral administration. *Drug Metabol. Dispos.* **20**, 909–911.
- Lauterwein M., Oethinger M., Belsner K., Peters T., and Marré R. (1995), *In vitro* activities of the lichen secondary metabolites vulpinic acid, (+)-usnic acid and (-)-usnic acid against aerobic and anaerobic microorganisms. *Antimicrob. Agents Chemother.* **39**, 2541–2543.
- Lawrey J. D. (1989), Lichen secondary compounds: evidence for a correspondence between antiherbivore and antimicrobial function. *Bryologist* **92**, 326–328.
- National Committee for Clinical Laboratory Standards (1993), Approved Standard M2-A5. Performance Standards for Antimicrobial Disk Susceptibility Tests. National Committee for Clinical Laboratory Standards, Villanova, Pennsylvania.
- Orange A., James P. W., and White F. J. (2001), *Microchemical Methods for the Identification of Lichens*. British Lichen Society, London.
- 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.
- Richardson D. H. S. (1988), Medicinal and other economic aspects of lichens. In: *CRC Handbook of Lichenology*, Vol. 3 (Galun M., ed.). CRC Press, Boca Raton, FL, pp. 93–108.
- Schumm F. (2002), Dünnschichtchromatogramme auch für den Amateur möglich. *Aktuelle Lichenologische Mitteilungen*, NF 9, Essen, pp. 8–22.
- Vartia K. O. (1973), Antibiotics in lichens. In: *The Lichens* (Ahmadjian V. and Hale M. E., eds.). Academic Press, New York, pp. 547–561.
- Yılmaz M., Özdemir Türk A., Tay T., and Kıvanç M. (2004), The antimicrobial activity of extracts of the lichen *Cladonia foliacea* and its (-)-usnic acid, atranorin, and fumarprotocetraric acid constituents. *Z. Naturforsch.* **59c**, 249–254.