The Antimicrobial Activity of Extracts of the Lichen *Cladonia foliacea* and Its (–)-Usnic Acid, Atranorin, and Fumarprotocetraric Acid Constituents

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The antimicrobial activity of the chloroform, diethyl ether, acetone, petroleum ether, and ethanol extracts of the lichen *Cladonia foliacea* and its (–)-usnic acid, atranorin, and fumarprotocetraric acid constituents against 9 bacteria and fungi has been investigated. The extracts and pure compounds alone were found active against the same bacteria and the same yeasts. *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Proteus vulgaris*, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Candida albicans*, and *Candida glabrata* growth were inhibited. In addition, the MICs of the extracts, (–)-usnic acid, atranorin and fumarprotocetraric acid were determined.

Key words: Cladonia foliacea, Antimicrobial Activity, Lichen Compound

Introduction

Lichens produce a wide range of organic compounds that can be grouped as primary metabolites and secondary metabolites (Elix, 1996). Primary metabolites such as proteins, lipids, carbohydrates, and some other organic compounds are needed for the lichen's metabolism and structure. Secondary metabolites are produced by the fungus alone and secreted onto the surface of lichen's hyphae either in amorphous forms or as crystals. About 350 secondary lichen metabolites have been found and the chemical structures of approximately 200 of them have been established (Galun, 1988). Lichen compounds are also known to show some biological activities against microorganisms. Lists of the antifungal and antibacterial activities of lichen compounds and lichens against bacteria and fungi can be found in a review and a book (Huneck, 1999, 2001).

Usnic acid is one of the most common and investigated lichen compounds. Its antimicrobial, antiprotozoal, antiviral, antiproliferative, anti-inflammatory, analgesic, antipyretic, and anti-tumour activities as well as some other properties such as UV protection, allergen, toxicity have been summarized in two recent reviews (Cocchietto *et al*., 2002; Ingólfsdóttir, 2002). It is a yellowish pigment and occurs in two enantiomeric forms, depending on the projection of the angular methyl group at the chiral 9b position. Fig. 1 gives the chemical structure of one of the enantiomers. Usnic acid and usnic acid containing lichens and their extracts have been utilized in medical, pharmaceutical, cosmetic and agricultural applications. Although atranorin and fumarprotocetraric acid are compounds found in many lichens, their antibacterial activities have not been studied in detail and little information about their biological activities is available in the literature (Ingólfsdóttir *et al*., 1998; Perry *et al*., 1999; see ref. 3 in Gudjónsdóttir and Ingólfsdóttir, 1997).

The early experiments on antibacterial activity of usnic acid go about 50 years back (Stoll *et al*., 1950). Ingólfsdóttir's review lists the antimicrobial activity of (+)- and (–)-usnic acid in a table against Gram-positive, Gram-negative, anaerobic bacteria, mycobacteria, and yeast/fungi with the relevant references (Ingólfsdóttir, 2002). The antibacterial activity of usnic acid against *Streptococcus mutans* has been examined by Ghione *et al*. (1988). 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*, and *Staphylococcus aureus*. Also it was reported that both forms of usnic acid inhibited the growth of *Mycobacterium tuberculo-*

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sis and *Mycobacterium tufu in vitro* at a relatively low concentration (Krishna and Venkataramana, 1992). The most common five lichen compounds were screened for *in vitro* activity against *Mycobacterium aurum* by Ingólfsdóttir *et al*. (1998). Among these compounds, (+)-usnic acid from *Cladonia arbuscula* exhibited the highest activity against *M. aurum* with a MIC value of 32 μg/ml. Atranorin, a depside and isolated from *Stereocaulon alpinum*, showed a MIC value of 250 μg/ml for the same microorganism. 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 and atranorin. 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 *Candida albicans* and *Trichophyton mentagrophytes*. No antimicrobial activity of atranorin (60 μg per disk) was detected against these microorganisms.

Experimental

Microorganisms

Staphylococcus aureus (ATCC 6538), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Aspergillus niger* (ATCC 9807), and *Fusarium solani* (ATCC 12820) (obtained from American Type Culture Collection/USA), *Proteus vulgaris* (NRRL B-123), *Bacillus cereus* (NRRL B-3711), *Bacillus subtilis* (NRRL B-744), and *Streptococcus faecalis* (NRRL B-14617) (obtained from Northern Regional Research Laboratory of the USDA, Peoria, Illinois/USA), *Listeria monocytogenes* (obtained from Ankara University, Faculty of Agriculture, Ankara, Turkey), *Yersinia enterocolitica* and *Aeromonas hydrophila* (obtained from Ankara University, Faculty of Veterinary, Turkey), *Pseudomonas syringae* pv. *tomato* (TPPB 4212), *Fusarium moniliforme*, *Fusarium oxyporum, Fusarium culmorum,* and *Rhizopus* sp., *Cladosporium* sp., and *Penicillium* sp. (obtained from Agriculture Research Center, Eskisehir/Turkey), *Candida albicans* and *Candida glabrata* (obtained from Osmangazi University, Faculty of Medicine, Eskisehir, Turkey), *Klebsiella pneumonia* and *Salmonella typhimirium* (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

Cladonia foliacea was collected from Eskisehir province in Turkey on the 4th of April, 2003. The collection site is in the east of Mayıslar village at 230 m. A herbarium sample is stored at the Herbarium of Anadolu University in the Department of Biology (ANES).

Extraction

For extraction, air-dried lichen sample was first ground, then 20 g portions were taken and added to 100 ml of solvents of diethyl ether, petroleum ether, chloroform, ethanol and acetone. The mixtures were sonicated for 30 min, then left at room temperature overnight. The extracts were filtered over Whatman No 1 filter paper, and the filtrates were sterilized by membrane filtration using 0.45 μm pore size filters.

Determination of antimicrobial activity

The Kirby and Bauer disk diffusion method (National Committee for Clinical Laboratory Standards, 1993) was used to determine the antimicrobial activity of lichen extracts against test bacteria and fungi. Bacteria strains were inoculated onto nutrient agar plate (10^8 cells/ml) and fungi strains were inoculated onto potato dextrose agar plate (10^8 spores/ml or cells/ml). The extracts for antimicrobial activity were screened by adding 5 ml of each extract in 1 ml portions to 50 filter paper disks (6 mm in diameter), allowing the solvent to evaporate between applications and leaving the lichen extracts on disks without the solvent. These disks were transferred into the inoculated microorganism media. Pure methanol, diethyl ether, petroleum ether, chloroform and acetone treated and dried disks served as negative control agents on the plates. Commercial chloramphenicol and ketoconazole were used as positive control substances. The bacterial plates were incubated for 24–48 h at 35–37 °C and the fungal plates for 5 d at 20–25 °C. Growth was evaluated visually by comparing the extract-containing disks with the control disks in a particular plate. The inhibition zones for bacteria and yeasts were

measured after 24–48 h. All experiments were done twice and checked with the control plates.

Determination of MICs of the extracts

MIC determinations of the extracts were carried out using the Kirby and Bauer disk diffusion method (National Committee for Clinical Laboratory Standards, 1993). After the solvents in the extracts had been evaporated using a rotary evaporator, the stock solutions of the residues were prepared by dissolving 20 mg of each residue in 2 ml of the same solvent in which the lichen extraction was performed (except in petroleum ether extract).

The stock solutions were subsequently diluted for two fold with pure solvents for fourteen times giving the final concentration range from 10 mg/ml to 0.61 μg/ml. Then 1 ml of each diluted solution was taken and added to 20 sterilized filter paper disks (6 mm in diameter) in a tube and the solvent was allowed to evaporate which eventually left the residue from the diluted solution on disk without solvent. An array of 0.5 mg–0.0305 μg residue-containing disks was obtained. For the residue from petroleum ether extract, 15 mg was taken and dissolved in 1.5 ml of petroleum ether.

Before transferring the disks into petri dishes, the microorganism media were prepared as follows: 250 μl (10^8 cells/ml) solutions of *Listeria monocytogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus cereus*, *Proteus vulgaris*, *Aeromonas hydrophila*, *Candida albicans*, and *Candida glabrata* were inoculated onto nutrient agar plates and onto potato dextrose agar plates, respectively. Then the disks containing an array of different amounts of residue were transferred on each plate. Negative and positive control agents used were the same as in the screening experiments. The bacterial and yeast plates were incubated for 24–48 h at 30–37 °C and minimum inhibition concentrations (MICs) were determined by checking the inhibition zones formed.

Bioautographic method using thin layer chromatography

After a preliminary thin layer chromatography (TLC) study, the acetone extract appeared to be the most concentrated solution among the extracts. Certain volumes of the acetone extract of *Cladonia foliacea* were taken and spotted on silica gel TLC plates (Merck, Silica gel 60 F_{254}), and then the TLC plates were developed in three solvent systems usually employed in the TLC of lichen substances. Solvent system A contained a mixture of toluene/dioxane/glacial acetic acid (36:9:1 v/v/v), solvent system B contained hexane/diethyl ether/formic acid (24:8:4 v/v/v), and solvent system C contained toluene/glacial acetic acid (20:3 v/v) (Culberson and Amman, 1979). Then, the developed plates were put into petri dishes covered with thin nutrient agar. Finally, soft nutrient agar including test microorganisms (10^8 cells/ml) was spread in 2 mm thickness to the petri dishes and these were incubated for 24–48 h at 35 °C. On the TLC plates, one substance showed antimicrobial activity and later it was identified as (–)-usnic acid. Two more substances were isolated from the acetone extract of *Cladonia foliacea* using preparative silica gel TLC. They were also identified as fumarprotocetraric acid and atranorin. The characterization of the three substances was based on checking their R_f values in different solvent systems with the ones given in the literature, their melting points and IR spectra (Huneck and Yoshimura, 1996; Culberson *et al.*, 1977; Orange *et al.*, 2001; Schumm, 2002; Edwards *et al.*, 2003). Also for fumarprotocetraric acid, the TLC of the acetone extract of *Cladonia coniocraea*, a control species for fumarprocetraric acid, was used as reference (Orange *et al.*, 2001). The enantiomeric form of usnic acid was determined using a polarimeter.

Determinations of MICs of (–)-usnic acid, fumarprotocetraric acid, and atranorin

The procedure described above for the determination of MICs of the extracts was applied to determine MICs of (–)-usnic acid, fumarprotocetraric acid, and atranorin. Stock solutions of (–)-usnic acid, fumarprotocetraric acid, and atranorin in acetone were prepared and subsequently diluted two fold. After treating the disks with the diluted solutions and evaporating the solvent of the solution, the amount of the substance on the disks varied from 2.5 mg to 0.076 μg for (–)-usnic acid, from 0.3 mg to 0.073 μg for fumarprotocetraric acid, and from 1.0 mg to 0.24 μg for atranorin.

Results and Discussion

We screened the antimicrobial activity of the chloroform, diethyl ether, acetone, petroleum

ether, and ethanol extracts of *Cladonia foliacea*. They were found active against 9 bacteria and fungi. All extracts showed or did not show antimicrobial activity against the same microorganisms. Bacteria *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Proteus vulgaris, Aeromonas hydrophila, Streptococcus faecalis,* and *Listeria monocytogenes* and the yeasts *Candida albicans* and *Candida glabrata* were the microorganisms whose growth were inhibited by the extracts. From these results, it could be concluded that Gram-positive bacteria are inhibited in general. Zone diameters were found roughly the same for each extract of *C. foliacea*. There was no antimicrobial activity of the extracts against the filamentous fungi tested and bacteria *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia*, *Salmonella typhimirium, Yersinia enterocolitica,* and *Pseudomonas syringae.* Before determining the MICs of the extracts, the residues extracted from 20 g of lichen with 100 ml solvent were recovered from the solvents using a rotary evaporator. The amounts of the residues were 90 mg for the chloroform extract, 223 mg for the acetone extract, 102 mg for the diethyl ether extract, 66 mg for the ethanol extract, and 15 mg for the petroleum ether extract. These results indicated that solvents with medium polarity such as acetone and diethyl ether were the proper solvents for the extraction of substances from *Cladonia foliacea*. Solvents with high polarity, like ethanol, and with very low polarity, like petroleum ether, did not extract efficiently. The MICs of the extracts were determined using the Kirby and Bauer method and the results are given in Table I.

We proceeded to determine antimicrobial active substances in an extract of *C. foliacea* as well as their MICs against the microorganisms and isolated three substances, (−)-usnic acid, fumarprotocetraric acid, and atranorin from the acetone extract. Although one of the substances, (−)-usnic acid, showed antimicrobial activity in the bioautographic method using TLC, fumarprotocetraric acid and atranorin also showed antimicrobial activity in the disk diffusion method. The reason why these two compounds did not show antimicrobial activity in the bioautographic method may be their low quantities, probably lower than their MICs, on

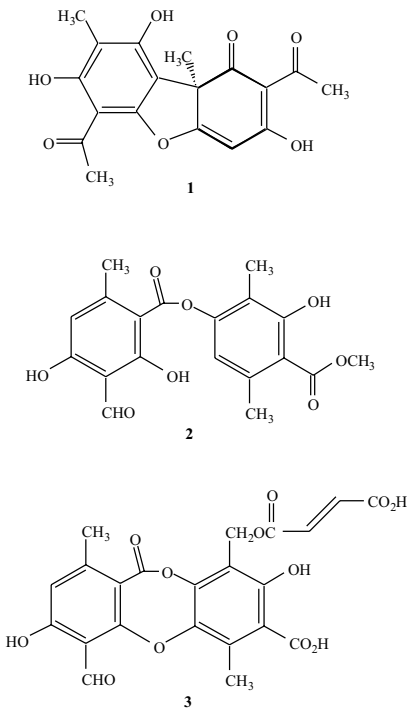

Fig. 1. Chemical structures of (−)-usnic acid (**1**), atranorin (**2**), and fumarprotocetraric acid (**3**).

Table I. MIC values of the extracts for bacteria and yeasts.

Microorganisms	MIC (against 10^7 cells) [μg]				
	Chloroform extract	Diethyl ether extract	Acetone extract	Petroleum ether extract	Ethanol extract
Staphylococcus aureus	0.97	15.6	7.8	0.73	3.9
Bacillus cereus	1.9	31.2	3.9	46.8	15.6
Bacillus subtilis	0.48	7.8	3.9	2.9	3.9
Proteus vulgaris	3.9	3.9	3.9	46.8	3.9
Aeromonas hydrophila	3.9	3.9	3.9	46.8	3.9
Streptococcus faecalis	0.24	0.97	3.9	0.73	0.97
Listeria monocytogenes	0.12	3.9	3.9	2.9	3.9
Candida albicans	500	500	500	375	500
Candida glabrata	500	500	500	375	500

the TLC spots. Table I shows the MIC values of the extracts for microorganisms resulted with antimicrobial activity.

The MIC values of (−)-usnic acid, atranorin and fumarprotocetraric acid are given in Table II. Each substance was found to be active alone against all of the microorganisms whose growth was inhibited by the extracts containing all or some of these compounds. In general, (−)-usnic acid was the most active one having remarkably low MICs. We obtained a MIC value of 0.15 μg (−)-usnic acid per disk against 10^7 cells for 3 bacteria and 2 yeasts. The high antimicrobial activity of usnic acid is known for a long time and our results show similar findings for the antimicrobial activity of (−)-usnic acid. Lauterwein *et al*. (1995) reported a MIC value range from 2 to 16 μg/ml for (−)-usnic acid against clinical isolates of *Staphylococcus aureus* (5×10^5 CFU/ml). Also they found that (−)-usnic acid was inactive against *Candida albicans* at concentrations $\leq$32 μg/ml. Perry and coworkers (1999) observed the antimicrobial activity of usnic acid against *Bacillus subtilis* and *Candida albicans*.

In our study, atranorin was the least active compound. Although no antimicrobial activity of atranorin against *Bacillus subtilis* and *Candida albicans* was found by Perry *et al*. (1999), we observed its activity and determined the MIC values of atranorin against these microorganisms (Table II). To the best of our knowledge no information about the antimicrobial activity of fumarprotocetraric acid is available for the last decade.

Moreover, there are reports on the inactiveness of (−)-usnic acid against *Escherichia coli* and *Pseudomonas aeruginosa* in the literature (Lauterwein *et al.*, 1995; Ingólfsdóttir, 2002). From our results obtained from the screening experiments, we may say that our finding is the same, although we did not test the pure (−)-usnic acid alone on these microorganisms.

Table II. MIC values of (−)-usnic acid, atranorin, and fumarprotocetraric acid from *Cladonia foliacea.*

Microorganisms	MIC (against 10^7 cells) [μg (mM)]		
	(−)-Usnic acid	Atranorin	Fumarprotocetraric acid
Bacillus cereus	0.15 (0.003)	31.2 (1.67)	4.6 (0.33)
Bacillus subtilis	0.61 (0.012)	15.6 (0.83)	4.6 (0.33)
Staphylococcus aureus	2.4 (0.046)	500 (26.7)	37.5 (2.65)
Streptococcus faecalis	0.15 (0.003)	250 (13.4)	150 (10.6)
Proteus vulgaris	0.15 (0.003)	62.5 (3.34)	37.5 (2.65)
Listeria monocytogenes	0.31 (0.006)	15.6 (0.83)	4.6 (0.33)
Aeromonas hydrophila	1.2 (0.023)	31.2 (1.67)	150 (10.6)
Candida albicans	0.15 (0.003)	500 (26.7)	18.7 (1.32)
Candida glabrata	0.15 (0.003)	500 (26.7)	18.7 (1.32)

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