

The Antimicrobial Activity of Extracts of the Lichen *Cetraria aculeata* and Its Protolichesterinic Acid Constituent

Ayşen Özdemir Türk^a, Meral Yılmaz^a, Merih Kıvanç^{a*}, and Hayrettin Türk^b

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

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

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

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

* Author for correspondence and reprint requests

Z. Naturforsch. **58c**, 850–854 (2003); received March 25/May 20, 2003

In this study, the antimicrobial activity of the acetone, diethyl ether and ethanol extracts of the lichen *Cetraria aculeata* has been investigated. The extracts were tested against twelve bacteria and eight fungi and found active against *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Proteus vulgaris*, *Streptococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*. No antimicrobial activity against the fungi was detected. It was determined that only one substance in the extracts has antimicrobial activity and it was characterized as protolichesterinic acid. The MICs of the extracts and protolichesterinic acid were also determined.

Key words: *Cetraria aculeata*, Protolichesterinic Acid, Antibacterial Activity

Introduction

Lichens produce a wide range of organic compounds that can be divided into two groups called primary metabolites and secondary metabolites (Elix, 1996). Primary metabolites are proteins, lipids, carbohydrates, and other organic compounds that are essential to the lichen's metabolism and structure. Some of these metabolites are produced by the lichen's fungal partner and others by the lichen's algal or cyanobacterial partners. Secondary metabolites are produced by the fungus alone and secreted onto the surface of lichen's hyphae either in amorphous forms or as crystals. If these substances are only found in lichens, then they are called lichen substances (Öztürk *et al.*, 1999).

The chemistry of about one third of all lichen species has been studied up to now and about 350 secondary metabolites are known from lichens. The chemical structures of approximately 200 of them have been established. They are extracellular products of relatively low molecular weight crystallized on the hyphal cell walls. Also they are usually insoluble in water and can be extracted into organic solvents. They amount to between 0.1 and 10 % of the dry weight of the thallus, sometimes up to 30 % (Galun, 1988).

After the discovery of penicillin from a fungus, numbers of lichens were screened for antibacterial

activity in the 1940s and 1950s (Vartia, 1973). Several lichen compounds were found active against Mycobacterium species and Gram-positive organisms. For example, usnic acid has been used as a topical antibacterial agent and also it showed antimicrobial activity against Gram-positive organisms *in vitro* (Stoll *et al.*, 1950; Lauterwein *et al.*, 1995). Protolichesterinic acid exhibited *in vitro* activity against *Helicobacter pylori* (Ingolfssdottir *et al.*, 1997). Lauterwein *et al.* (1995) investigated *in vitro* activities of vulpinic acid and usnic acid against some aerobic and anaerobic microorganisms. Fournet *et al.* (1997) studied the activity of the lichen compounds usnic acid, pannarine and 1'-chloropannarine against promastigotes forms of three strains of *Leishmania* spp. In addition lichens have been used for medicinal purposes throughout centuries. For example, *Lobaria pulmonaria*, *Cetraria islandica*, and *Cladonia* species were reputed to be effective in the treatment of pulmonary tuberculosis (Vartia, 1973).

In this research, we investigated the extracts of *C. aculeata* for antimicrobial activity on twelve bacteria and eight fungi and found that protolichesterinic acid obtained from *C. aculeata* is the substance responsible for the antimicrobial activity against some of the tested bacteria. *C. aculeata* is the most common terricole *Cetraria* species in Turkey. It is al-

readily established that protolichesterinic acid, an α -methylene- γ -lactone (Fig. 1), is the major biologically active secondary metabolite in the lichen *Cetraria islandica*, known as Iceland moss (Culberson *et al.*, 1977; Ingólfssdóttir *et al.*, 1997).

Experimental

Organisms

Twelve bacterial strains and eight fungal strains listed below were used to assess the antimicrobial

properties of the test samples. Bacterial strains and fungal strains were maintained in the 15 % glycerol at -80°C .

Overnight bacterial cultures were prepared by inoculating 5 ml BHI broth with one loop of each bacterial organism taken from NA (nutrient agar). Broth was incubated overnight at 35°C . Fungi were incubated on plate count agar for 5–7 days at 25°C .

Test bacteria

<i>Bacillus cereus</i>	NRRL B-3711
<i>Staphylococcus aureus</i>	NRRL B-767
<i>Escherichia coli</i>	NRRL B-3704
<i>Proteus vulgaris</i>	NRRL B-123
<i>Pseudomonas aeruginosa</i>	NRRL B-23
<i>Streptococcus faecalis</i>	NRRL B-14617
<i>Bacillus subtilis</i>	NRRL B-744
<i>Pseudomonas syringae</i>	TPPB 4212
<i>Aeromonas hydrophila</i>	
<i>Yersinia enterocolitica</i>	
<i>Listeria monocytogenes</i>	
<i>Klebsiella pneumoniae</i>	

Test fungi

<i>Penicillium sp.</i>	
<i>Cladosporium sp.</i>	
<i>Fusarium oxysporum</i>	
<i>Fusarium culmorum</i>	
<i>Rhizopus sp.</i>	
<i>Fusarium moniliforme</i>	
<i>Fusarium solani</i>	ATCC 12820
<i>Aspergillus sp.</i>	ATCC 9807

Bacteria supplier

Northern Regional Research Laboratory of the USDA
Northern Regional Research Laboratory of the USDA
Northern Regional Research Laboratory of the USDA
Northern Regional Research Laboratory of the USDA
Northern Regional Research Laboratory of the USDA
Northern Regional Research Laboratory of the USDA
Northern Regional Research Laboratory of the USDA
Agriculture Research Center, Eskisehir/Turkey
Faculty of Veterinary, Ankara University/Turkey
Faculty of Veterinary, Ankara University/Turkey
Faculty of Veterinary, Ankara University/Turkey
Department of Biology, Anadolu University/Turkey

Fungi supplier

Department of Biology, Anadolu University/Turkey
Department of Biology, Anadolu University/Turkey
Faculty of Agriculture, Çukurova University/Turkey
Faculty of Agriculture, Çukurova University/Turkey
Department of Biology, Anadolu University/Turkey
Agriculture Research Center, Eskisehir/Turkey
American Type Culture Collection/USA
American Type Culture Collection/USA

Lichen material

Cetraria aculeata was collected from Eskisehir province in Turkey on the 21st of September 2002. The collection site is in Bozdağ, east of Tandır village at 1150 m. Herbarium sample of the material is stored at the Herbarium of Anadolu University in the Department of Biology (ANES).

Extraction from lichen sample

For extraction, lichen sample was first ground, then 10 g portions were taken and added to 100 ml of solvents of diethyl ether, 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. The filtrates were sterilized by membrane filtration using $0.45\ \mu\text{m}$ pore size filters.

Determination of antimicrobial activity

The antimicrobial activity of lichen extracts against test bacteria and fungi was determined according to the Kirby and Bauer disk diffusion method (National Committee for Clinical Laboratory Standards, 1993). Bacteria strains were inoculated onto nutrient agar plate (10^8 cells/ml) whereas fungi strains were inoculated onto potato dextrose agar plate (10^8 spores/ml). Test solutions were screened by adding 4 ml of lichen extracts to 80 filter paper disks (6 mm in diameter), allowing the solvent to evaporate between applications and leaving the lichen extracts on per disk without the solvent. Pure methanol, diethyl ether and acetone served as negative control agents on the plates. Commercial bactericide chloramphenicol and fungicide ketoconazole were used as positive control substances. The bacterial plates were incubated for

24–48 h at 35–37 °C and the fungal plates were incubated for 5 days at 20–25 °C. Growth was evaluated visually by comparing a particular plate with the control plates. The inhibition zones for bacteria were measured after 48 h. All experiments were done twice and checked with the control plates.

MIC determination

Minimum inhibitory concentration (MIC) for bacterial growth was determined by a serial dilution technique using 96-well microtitre plates. MICs were calculated for the test bacteria only that had antimicrobial activity.

Amount of substance used in MIC determination was calculated after evaporating the solvent of 1 ml of extract and then solubilizing the dry extract in 20 % v/v dimethyl sulfoxide (DMSO). The solution was subsequently diluted for 10 fold with water up to 10⁻¹⁰ dilution. 100 µl from both bacterium solutions and dilutions were transferred into microtitration plates and incubated for 24–48 h at 35–37 °C. One of the positive controls contained 100 µl of bacterium solution plus 20 % DMSO solution in a well and another one had 100 µl of bacterium solution plus 100 µl nutrient broth in a well. Third control was chloramphenicol (62.5 µg/ml) for each bacterium. Negative controls contained only dilute solutions without bacteria. Positive and negative results were evaluated according to turbidity occurred after 24–48 h by comparing to the ones in the control wells. The lowest concentrations giving no visible growth for each bacterium were defined as MIC. To determine the antibacterial activity of whether it is bacteriostatic or bacteriocidal, 10 µl constituents from each single well (1 to 12) were dropped to nutrient agar plates and the plates were incubated for 24 h. In addition to above controls, bacterial growth plates including 10 µl test bacteria were also incubated for 24 h. If the growth is not observed on the nutrient agar but present on the control plates, the activity is bacteriocidal, otherwise it is bacteriostatic (Elof, 1998).

Bioautographic method using thin layer chromatography

Having determined MICs, a certain volume of acetone extract of *Cetraria aculeata* was spotted on

silica gel thin layer chromatography (TLC) plates (Merck, Silica gel 60 F₂₅₄) 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), the solvent system B contained hexane/diethyl ether/formic acid (24:18:4 v/v), the solvent system C contained toluene/glacial acetic (20:3 v/v) (Culberson and Aman, 1979). Then, the developed plates were put into petri dishes covered with thin nutrient agar. Finally, soft Muller Hilton Agar including test microorganisms (10⁸ cells/ml) was spread over 2 mm thickness to the petri dishes and the petri dishes were incubated for 24–48 h at 35 °C. After that, the substance showing antimicrobial activity was determined (Cannel, 1998). That particular substance isolated from acetone extract of *C. aculeata* was protolichesterinic acid and purified using silica gel preparative TLC. It was characterized by checking its R_f values in different solvent systems with the ones giving in the literature and its melting point (Schumm, 2002) as well as with the TLC of the acetone extract of *C. islandica*, commonly known as Iceland moss. This pure protolichesterinic acid was used to determine its antimicrobial activity against *E. coli*, *B. subtilis*, *P. aeruginosa*, *L. monocytogenes*, and its MIC values against these bacteria were also determined as describe above.

Results and Discussion

We found that the *Cetraria aculeata* extracts showed antimicrobial activity against most of the tested bacteria, except the ones *P. syringae*, *K. pneumoniae*, *Y. enterocolitica*. Zone diameters were found roughly the same for each extract of *C. aculeata*. In addition there was no antimicrobial activity of the extracts against all fungi tested.

Table I shows the MIC values of the extracts for bacteria resulted with antimicrobial activity. After the evaluation of the results, the acetone extract of *C. aculeata* showed the highest inhibitory activity among the other extracts against the bacteria of which their growth is inhibited. The ethanol and diethyl ether extracts were slightly less active compared to that of the acetone extract. For the acetone extract, the antibacterial was detected at a

Microorganism	MIC (against 10 ⁷ cells) [μ g/ml]		
	Ethanol extract	Diethyl ether extract	Acetone extract
<i>E. coli</i>	1215	2330	850
<i>S. aureus</i>	1215	1165	425
<i>A. hydrophila</i>	607	2330	212
<i>P. vulgaris</i>	6050	8460	1480
<i>S. faecalis</i>	6050	8460	1480
<i>B. cereus</i>	3025	8460	2960
<i>B. subtilis</i>	6050	8460	1480
<i>P. aeruginosa</i>	3025	8460	1480
<i>L. monocytogenes</i>	3025	8460	741.5

Table I. MIC values of the extracts for bacteria.

concentration as low as 212 μ g/ml (against 10⁷ *A. hydrophila* cells). We think that acetone is the more suitable solvent for the extraction than diethyl ether and ethanol. Eventually the acetone extract contains more microbial active substance and has lower MIC values.

In addition we observed bacteriocidal activity against *P. vulgaris*, *S. faecalis*, *B. cereus*, *B. subtilis*, *L. monocytogenes* and the activity was bacteriostatic against *E. coli*, *S. aureus*, *A. hydrophila*, *P. aeruginosa*.

The obtained MIC values in this study appear to be higher than the MIC values reported in the literature. However, the number of microorganisms used in this study is about 100–1000 times higher, 10⁷ cells/ml with respect to 10⁴–10⁵ cells/ml (Ingolfsson *et al.*, 1997). So, we think the MIC values obtained in this study are reasonable.

After finding the antibacterial activity and calculating the MIC values of the extracts of *C. aculeata*, the antibacterial active substance has been determined using the bioautographic method. Only one substance in the extracts was found to be active. The position of the spot showing inhibition zone was different on each TLC plate depending on the TLC development solvent system. We isolated this substance using preparative TLC, characterized it using its R_f values and melting point, and identified it as protolichesterinic acid (Fig. 1).

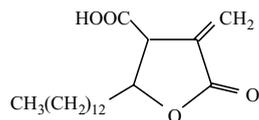


Fig. 1. Chemical structure of protolichesterinic acid.

Protolichesterinic acid was also isolated from *C. islandica* known as Iceland moss before and its antimicrobial activity has been known for a long time. In 1950s, it was found that it is an antimicrobial active substance against *M. tuberculosis*, *S. pyrogenes*, and *S. aureus*. In a more recent investigation, light petroleum extracts of Iceland moss were found active against *S. aureus*, *B. subtilis*, and *Candida albicans*. Protolichesterinic acid has further been shown to exhibit antitumor activity against solid-type Ehrlich carcinoma in mice, potent *in vitro* inhibiting activity of against the DNA polymerase activity of human immunodeficiency virus type 1 reverse transcriptase, and inhibitory effects on arachidonate 5-lipoxygenase from porcine leukocytes (Ingolfsson *et al.*, 1997).

We also determined the MIC values of protolichesterinic acid alone against *E. coli*, *B. subtilis*, *P. aeruginosa*, *L. monocytogenes* and the results are given in Table II. Furthermore its antimicrobial activity against *B. subtilis* and *L. monocytogenes* was bacteriosidal and that against *E. coli* and *P. aeruginosa* was bacteriostatic as in the acetone extract of *C. aculeata*.

The antimicrobial activity of protolichesterinic acid against *H. pylori* and *M. aurum* has already been reported in the literature (Zechmeister,

Table II. MIC values of protolichesterinic acid for bacteria.

Microorganism	MIC (against 10 ⁷ cells)	
	[μ g/ml]	[mm]
<i>E. coli</i>	7341	22.6
<i>B. subtilis</i>	7341	22.6
<i>P. aeruginosa</i>	7341	22.6
<i>L. monocytogenes</i>	3670	11.3

2001). This report concludes that protolichesterinic acid is also active against *E. coli*, *B. subtilis*, *P. aeruginosa*, *L. monocytogenes*.

Although it looks promising to use the secondary metabolites of *C. aculeata* or protolichesterinic

acid in food and pharmaceutical industries, further investigations on the antimicrobial activity as well as the economical and fast isolation of the metabolite from the lichen are needed.

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