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In Vitro Susceptibility of *Helicobacter pylori* to Protolichesterinic Acid from the Lichen *Cetraria islandica*

KRISTIN INGOLFSDOTTIR,[1]* MARTHA A. HJALMARSDOTTIR,[2] AXEL SIGURDSSON,[3] GUDBORG A. GUDJONSDOTTIR,[1] ASA BRYNJOLFSDOTTIR,[1] AND OLAFUR STEINGRIMSSON[2]

Department of Pharmacy, University of Iceland, 107 Reykjavik,[1] and Department of Microbiology, National University Hospital,[2] and National University Hospital Pharmacy,[3] 101 Reykjavik, Iceland

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With reference to the traditional use of *Cetraria islandica* (Iceland moss) for relief of gastric and duodenal ulcer, plant extracts were screened for in vitro activity against *Helicobacter pylori*. (+)-Protolichesterinic acid, an aliphatic α-methylene-γ-lactone, was identified as an active component. The MIC range of protolichesterinic acid, in free as well as in salt form, was 16 to 64 μg/ml.

Following the discovery of penicillin production by the fungus *Penicillium notatum*, a number of lichens, which consist of a symbiotic association between an algal and a fungal partner, were screened for antibacterial activity between 1940 and 1950 (15). Several lichen compounds were found active against mycobacteria and gram-positive organisms (14, 15). The lichen *Cetraria islandica* (L.) Ach., commonly known as Iceland moss, has been used in European traditional medicine for treatment of minor ailments such as throat irritation and cough, but also for tuberculosis, asthma, and gastrointestinal conditions such as gastritis (8). In Iceland the plant has furthermore been used for symptomatic relief of gastric and duodenal ulcer.

Scientific investigations of the biological activity of Iceland moss constituents include in vitro and in vivo studies on antitumor and immunostimulating properties of polysaccharides found in the plant (1, 6). In a study from 1950, protolichesterinic acid from Iceland moss was found to possess antibacterial properties against *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* (14). In a more recent investigation, light petroleum extracts of Iceland moss were found active against *S. aureus*, *Bacillus subtilis*, and *Candida albicans* (5). Protolichesterinic acid has further been shown to exhibit antitumor activity against solid-type Ehrlich carcinoma in mice (3), potent in vitro inhibiting activity against the DNA polymerase activity of human immunodeficiency virus type 1 reverse transcriptase (12), and inhibitory effects on arachidonate 5-lipoxygenase from porcine leukocytes (7).

With reference to the use of Iceland moss to relieve symptoms of gastric and duodenal ulcer, an investigation was undertaken whereby extracts of the lichen were screened for in vitro inhibitory activity against *Helicobacter pylori*, the organism reputed to contribute to the etiology of gastritis as well as gastric and duodenal ulcer (9). *H. pylori* has further been suggested as a risk factor for certain forms of gastric cancer (4).

Cetraria islandica was collected in a mountain range in the eastern part of Iceland (Jökuldalsheidi) and cleansed of extraneous material prior to grinding. For primary screening, small samples (10 g) of ground plant material were successively extracted in a Soxhlet apparatus with solvents of increasing polarity: light petroleum boiling range 40 to 60°C (E. Merck, Darmstadt, Germany, no. 915), acetone (Merck no. 2500), methanol (Merck no. 6009), and water. Organic extracts were evaporated to dryness under reduced pressure; the water extract was freeze-dried.

The dried organic extracts were dissolved in methanol at maximum permissible concentrations for the preparation of test solutions: light petroleum extract (0.6 mg/ml), acetone extract (0.9 mg/ml), and methanol extract (1.5 mg/ml). A test solution of the water extract was prepared by dissolving the freeze-dried extract in water at a concentration of 7.0 mg/ml.

For diffusion susceptibility tests four strains of *H. pylori*, with different antibiograms, were used. The bacterial strains were kept in tryptic soy broth with 20% glycerol at −70°C. The stock cultures were subcultured twice prior to testing on blood agar (heart infusion agar [Oxoid, Basingstoke, United Kingdom] supplemented with 5% defibrinated horse blood) incubated at 37°C under microaerophilic conditions (85% N_2, 10% CO_2, 6% O_2; Oxoid gas-generating kit and GasPak jars; BBL Microbiology Systems, Cockeysville, Md.) with CampyPak Plus (BBL).

The Kirby and Bauer disk diffusion susceptibility test was used for primary screening of susceptibility of *H. pylori* to the plant extracts. The tests were performed according to recommendations of the National Committee for Clinical Laboratory Standards (10), modified to the needs of *H. pylori*. The *H. pylori* strains were inoculated onto Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% defibrinated horse blood. The test solutions were screened by adding 300 μl of methanol solutions to Bacto (Difco) filter paper disks (12.7 mm in diameter) in 30-μl portions, allowing the solvent to evaporate between applications (final concentration per disk: petroleum extract, 180 μg; acetone extract, 270 μg; methanol extract, 450 μg). Pure methanol served as a control. The applied volume of the water extract was 30 μl per disk (final concentration per disk, 2.1 mg). The plates were incubated at 37°C under microaerophilic conditions (85% N_2, 10% CO_2, 6% O_2; Oxoid gas-generating kit and GasPak jars with CampyPak Plus).

Results, evaluated after 48 to 72 h, showed that the light petroleum extract exhibited inhibitory activity. The acetone extract was slightly active, but methanol and water extracts were inactive. The active compound in the light petroleum extract was identified as (+)-protolichesterinic acid, an ali-

* Corresponding author. Mailing address: Dept. of Pharmacy, University of Iceland, Hagi/Hofsvallagata, 107 Reykjavik, Iceland. Phone: 354-525-4000. Fax: 354-525-4071. E-mail: kring@rhi.hi.is.

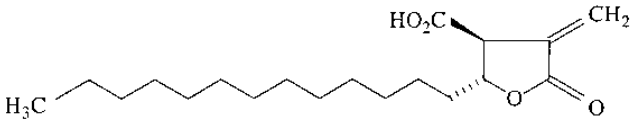

FIG. 1. Chemical structure of protolichesterinic acid, an aliphatic α-methylene-γ-lactone from *C. islandica.*

phatic α-methylene-γ-lactone (Fig. 1). When a petroleum extract, from which protolichesterinic acid had been largely removed by fractional precipitation, was tested, the extract was no longer active. Protolichesterinic acid is also considered responsible for the slight activity of the acetone extract, as it is present in small amounts in this extract; i.e., it is not exhaustively extracted by light petroleum.

Qualitative screening of protolichesterinic acid was performed using the Kirby and Bauer disk diffusion test as before with a methanolic test solution containing 1.0 mg/ml. Protolichesterinic acid was isolated and identified through chromatographic and spectroscopic analysis as previously described (7).

For determinations of MICs of protolichesterinic acid, *H. pylori* ATCC (American Type Culture Collection) 43504 and 30 bacterial strains, randomly selected from human gastroscopic biopsy samples collected at the National University Hospital from 1992 to 1995, were used in addition to the four strains used for primary screening. The bacterial strains were maintained as described above. MIC determinations were performed with agar dilution according to recommendations of the National Committee for Clinical Laboratory Standards (11), modified for testing *H. pylori*. In order to eliminate solubility problems and the use of organic solvents, a sodium salt solution of protolichesterinic acid in water (10 mg/ml) was prepared. Double dilutions of this solution in 1-ml aliquots were added to 19 ml of melted Mueller-Hinton agar supplemented with 5% defibrinated horse blood. Final concentrations in the agar of sodium protolichesterinate ranged from 0.008 to 512 μg/ml. Bacteria were inoculated onto the plates with a multipoint replicator (Mast Laboratories Ltd., Liverpool, United Kingdom) which delivered an inoculum of approximately 10^4 CFU. The plates were incubated at 37°C under microaerophilic conditions (85% N_2, 10% CO_2, 6% O_2; Oxoid gas-generating kit and GasPak jars with CampyPak Plus). The MIC was determined after an incubation period of 48 to 72 h.

The MIC range for sodium protolichesterinate proved to be 16 to 64 μg/ml (Table 1). The MIC value for the standard strain (ATCC 43504) was 16 μg/ml. The MIC_{90} value, i.e., the MIC at which 90% of the isolates are inhibited, was 32 μg/ml.

In order to study whether the activity of the salt differed from that of the free acid, an MIC determination for protolichesterinic acid in dimethylsulfoxide (Merck no. 2950) was performed as above using a stock solution of 10 mg/ml. Although dimethylsulfoxide variably affects the growth of *H. pylori*, it was possible, through repeated experiments, to show that the MIC range for protolichesterinic acid is consistent with MIC results for the sodium salt solution.

TABLE 1. In vitro susceptibility of 35 strains of *H. pylori* to sodium protolichesterinate

Strain(s)[a]	No. of isolates	No. of strains for which MIC (μg/ml) was:		
		16	32	64
Clinical isolates	34	2	30	2
ATCC 43504	1	1		

[a] MIC_{90}, 32 μg/ml.

The present results confirm the presence of a compound in Iceland moss capable of suppressing in vitro growth of *H. pylori* and are of interest with respect to the use of the plant to relieve symptoms of gastric and duodenal ulcer. The MIC_{90} observed for protolichesterinic acid (32 μg/ml) is considerably higher than that exhibited by antibacterial agents such as ampicillin (0.125 μg/ml) and erythromycin (0.25 μg/ml), using the same assay procedure (2), but only twice as high as that of metronidazole (16 μg/ml). Whether such a comparison is relevant as a guide to the in vivo effectiveness of protolichesterinic acid, however, will be a subject for further study. To our knowledge this is the first report of the screening of lichen constituents for activity against *H. pylori.*

H. pylori is a fastidious, slow-growing organism sensitive to changes in pH of the culture media. The fact that the sodium salt of protolichesterinic acid is as inhibitory as the free acid indicates that the activity is not obtained through a lowering of pH.

It has previously been shown by high-pressure liquid chromatographic analysis that despite its lipophilic nature, protolichesterinic acid can be directly extracted into aqueous media and is present in Iceland moss preparations produced by traditional methods, i.e., by boiling the plant material directly with water (7). This is most likely due to solubilizing effects of other components present in such extracts.

The reputed beneficial effects of Iceland moss in cases of gastritis and gastric and duodenal ulcer could be due in part to inhibitory activity of protolichesterinic acid against *H. pylori*. It could further be speculated that other reported effects of protolichesterinic acid are contributive, most notably in vitro inhibitory activity against 5-lipoxygenase (7). Lipoxygenase products, i.e., leukotrienes, have been implicated as mediators of inflammatory responses in the gastrointestinal tract (13). The exact mode of action of protolichesterinic acid will obviously remain speculative until further investigations have been carried out.

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