

Short communication

Antimicrobial activity of some coumarin containing herbal plants growing in Finland

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Abstract

Antimicrobial screening against selected Gram-positive and Gram-negative bacteria, yeasts, mold, as well as plant pathogenic fungi, with emphasis on method optimization was carried out on methanol extracts prepared from seven plants grown in Finland. Sensitivity to the extracts was found to vary considerably among the micro-organisms, the extract from *Petroselinum crispum* and *Ruta graveolens* showing the highest toxicity against *Rhizoctonia solani*. The growth of *Heterobasidium annosum* was inhibited, whereas that of *Phytophthora (cactorum)* was promoted by all the extracts. The antibacterial and antifungal activities of six natural coumarin compounds were weak, except for the inhibitory effect against *Fusarium culmorum*. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents (Press, 1996), as well as serving drug discovery from natural products for

primary lead compounds (Lawrence, 1999). Traditional antibacterial therapy is going through a crisis due to the rapidly increasing development of resistance to existing agents. Such resistance has an impact on all areas of chemotherapy. The first pathogen (*Staphylococcus aureus*) that has become resistant to all known antibiotics has posed a threat already for a number of years. It has thus become apparent that new antimicrobial agents will continue to select for resistant strains from the pool of bacteria which continuously

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undergo genetic change. Besides possible antimicrobial properties against human pathogens, it is also of interest to know whether these extracts or compounds possess activity against plant pathogens. Agents for biocontrol are of interest in Finland especially for the forestry looking for natural solutions.

As a part of a research program directed at the isolation of active coumarins from plants grown in Finland, this paper describes the preliminary investigations on the antimicrobial activity of seven plant crude extracts and six pure natural coumarins found in these plants, with special emphasis on methodology.

2. Methodology

2.1. Plant extracts

The Apiaceae plants studied were *Aegopodium podagraria* L. (herbarium number As3-5), *Anethum graveolens* L. (As5-5), *Angelica archangelica* L. (As7-5), *Levisticum officinalis* Koch (As10-5), *Petroselinum crispum* (P. Mill.) A. W. Hill. (As11-5), and *Peucedanum palustre* (L.) Moench (L4-2). The plant belonging to the Rutaceae family was *Ruta graveolens* L. (As13-5). The plants were supplied by Dr B. Galambosi (senior research scientist at the Agricultural Centre, Mikkeli, Finland) in summer 1995, except for *P. palustre* which was collected from Laajalahti, Espoo, Finland (identified by Docent K. Fagerstedt, Division of Plant Physiology, Department of Biosciences, University of Helsinki, Finland) in 1992. Voucher specimens are deposited at the Division of Pharmacognosy, Department of Pharmacy, University of Helsinki, Finland. Air-dried and powdered plant material (about 20.0 g) was extracted three times with methanol (proportions 133 + 67 + 67 ml; Rathburn, UK), sonicated for 15 min, and centrifuged (600 × g, 5 min). Repeatability of the extraction process for total coumarins was 9.6 c.v.% ($n = 6$), calculated from the bergapten standard curve. Chemical analysis of the combined extracts was performed by a method developed by Vuorela et al. (1989). Bergapten (Fluka Chemie AG, Switzerland)

served as the standard compound. Combined extracts from the three consecutive extractions were lyophilized and dissolved in methanol (1 mg/ml). Five hundred micrograms of each sample was added to the inoculated agar dishes either absorbed on paper discs or as a solution applied into a hole.

2.2. Coumarins

Bergapten (Fluka Chemie AG, Switzerland), coumarin (E. Merck KGaA, Germany), herniarin, umbelliferone and xanthotoxin (all from Carl Roth GmbH, Germany), and scopoletin (Sigma, St. Louis, MO) were dissolved in a suitable solvent (methanol or acetone) to give a concentration of 1 mg/ml. Five hundred micrograms of each sample was added to the inoculated agar dishes.

2.3. Bacteria, yeasts and mold

Strains of bacteria, yeasts and mold, their cultivation media used in the study and the positive controls were *Bacillus subtilis* [ATCC 9372 (American Type Culture Collection, USA); nutrient agar; tetracyclin/ + + + (see Table 1 for interpretations)], *Escherichia coli* (ATCC 8739; nutrient agar; sulphonamide/ + + +), *Micrococcus luteus* [YMBO (Division of General Microbiology, University of Helsinki, Finland); seed agar; bacitracin/ neomycin/ + + +], *Pseudomonas aeruginosa* (ATCC 9027; nutrient agar; ampicillin/ + + +), *S. aureus* [DSM 20231 (Deutsche Sammlung von Micro-organismen, Germany); nutrient agar; penicillin/ + + +], *Staphylococcus epidermidis* (ATCC 12228; nutrient agar; ampicillin/ + + +), *Candida albicans* (ATCC 10231; Sabouraud agar; amphotericin B/ + +), *Saccharomyces cerevisiae* [FOMK (Division of Pharmacognosy, University of Helsinki, Finland); Sabouraud agar; amphotericin B/ + +], and *Aspergillus niger* [ATCC 16404; Sabouraud agar and potato dextrose agar (PDA); amphotericin B/ –].

Neo-sensitabs (A/S Rosco Diagnostica, Denmark) preloaded with a constant amount of diffusible antimicrobial agent [penicillin (5 µg), ampicillin (33 µg), tetracyclin (80 µg),

Table 1
Antimicrobial effects of leaf extracts and coumarins on bacteria, yeasts and mold^a

Extracts (coumarin content) Coumarins	Microbial species									
	<i>B. subtilis</i>	<i>E. coli</i>	<i>M. luteus</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>C. albicans</i>	<i>S. cerevisiae</i>	<i>A. niger</i>	
<i>A. podagrarica</i> (5.0 µg/ml)	(+)	–	–	+	+	+	–	–	–	
<i>A. graveolens</i> (5.4 µg/ml)	(+)	–	–	+	+	+	–	–	–	
<i>A. archangelica</i> (389 µg/ml)	(+)	–	–	+	+	+	–	–	–	
<i>L. officinalis</i> (79 µg/ml)	(+)	–	–	+	+	+	–	–	–	
<i>P. crispum</i> (37 µg/ml)	(+)	–	–	+	+	+	–	+	–	
<i>P. palustre</i> (834 µg/ml)	(+)	–	–	–	+	–	–	–	–	
<i>R. graveolens</i> (126 µg/ml)	(+)	–	+	+	+	+	–	–	–	
Bergapten	–	–	–	–	–	–	–	–	–	
Coumarin	–	(+)	–	+	+	–	–	–	–	
Herniarin	–	–	–	–	–	–	–	–	–	
Scopoletin	–	–	–	–	+	–	–	–	–	
Umbelliferone	–	(+)	–	+	–	(+)	–	–	–	
Xanthotoxin	(+)	–	–	–	(+)	–	–	+	+	
Control (methanol, acetone)	–	–	–	–	–	–	–	–	–	

^a Sample amount 500 µg ($n = 3-6$). –, no antimicrobial activity, inhibition zone of sample < inhibition zone of methanol + 1 mm; +, a modest antimicrobial activity, i.z. of sample 1–4 mm > i.z. of methanol; ++, a clear antimicrobial activity, i.z. of sample 4–10 mm > i.z. of methanol; ++++, a strong antimicrobial activity, i.z. of sample > i.z. of methanol + 10 mm; () the effect is only bacteriostatic.

sulphonamide (240 µg)], as well as bacitracin (10 U, Ph.Nord.), neomycin sulfate (100 µg, Ph.Nord.) and amphotericin B (225 µg, Sigma) served as the positive controls. The solvent was used as the negative control.

The medium consisted of two layers, only the upper layer being inoculated. Thirty ml of sterile base agar (Difco, MI, USA) was poured into Sterilin petri dishes (ø = 14 cm, Bibby Sterilin, UK). The agar was left to set, after which 30 ml of Isosensitest agar (OXOID, UK) or Sabouraud agar [self-prepared and used for *C. albicans*, *S. cerevisiae*, *A. niger*; glucose (Ph.Eur.) 40.0 g, peptone (Biokar Diagnostics, France) 10.0 g, agar (Biokar Diagnostics, France), 20.0 g, distilled water ad 1000.0 ml] was poured on top. The Isosensitest agar consisted of a 24.2 µl of bacterial suspension in 30 ml, and the Sabouraud agar of 250 µl of yeast or mold suspension in 30 ml. Culturing was stopped when the density of the microbial suspensions had reached an absorbance of 0.8–1.0 measured at 625 nm with a spectrophotometer (Ultraspec III, Pharmacia, Sweden). The suspensions were diluted with sterile isotonic sodium chloride solution to an absorbance of 0.1 at 625 nm (Barry and Thornsberry, 1991).

Prior to incubation in darkness at +35°C (or +22°C for fungi) for 24 h, there was a 1 h period of diffusion at room temperature (or +8°C for fungi). After incubation the mean of two diameters with a 0.1 mm precision was calculated, and the effect was evaluated as partial or total inhibition.

2.4. Plant pathogenic fungi

Strains of plant pathogenic fungi, their cultivation media used in the study and the positive controls were *Botrytis cinerea* [YMBO; PDA; amphotericin B/+ (see Table 2 for interpretations)], *Fusarium culmorum* (YMBO; PDA; amphotericin B/+ + +), *Heterobasidium annosum* [METLA 94265 (Finnish Forest Research Institute, Finland), isolated from root of *Pinus sylvestris*; malt agar; amphotericin B/+ +], *Phytophthora (cactorum)* PH5 [METLA (unpublished identification, Arja Lilja), isolated from root of *Betula pendula*; malt agar; amphotericin B/+ +], *Rhizoctonia solani* (YMBO; PDA; amphotericin B/+ +), and *Rhi-*

zoctonia uni-nucleate strain (264) (YMBO, isolated from bare-rooted seedlings of *Pinus sylvestris*; PDA; amphotericin B/+ +). The solvent was used as the negative control.

Three of the six plant pathogens chosen for the study, *H. annosum*, *Phytophthora (cactorum)* PH5 and *Rhizoctonia* uni-nucleate strain (264), were originally isolated from *Pinus sylvestris* (Scots pine) and *Betula pendula* (birch) trees growing in Finland (Elo et al., 1997). *B. cinerea* is an unspecified plant pathogenic fungi which causes grey mold on a wide variety of host plants, e.g. strawberries. *Fusarium* and *Rhizoctonia* cause wilt diseases on American ginseng (Punja, 1997). *Heterobasidium* induces root rot on spruce, pine and birch, and it is a destructive root pathogen of conifers, whereas necrotic areas on the leaves and stems of birch can be caused by *Phytophthora*.

The test was started by inoculating an agar dish with a piece of fresh mycelium (diameter 12.5 mm). The medium consisted of two layers, only the upper layer being inoculated. 30 ml of sterile base agar was poured into petri dishes (ø = 14 cm). The agar was left to set, after which 30 ml of potato-dextrose-agar [used for all except *H. annosum*, *Phytophthora (cactorum)* PH5] or malt agar (both from Pronadisa, Hispanlab S.A., Spain) was poured on top.

When the inoculum had grown for a distance of 2 cm, the discs were placed on the agar at a distance of 1.5–2 cm from the edge of the inoculum. The dishes were kept at +8°C for 1 h for prediffusion of the samples, and then incubated in darkness at room temperature. Evaluation was performed daily, and the final results were recorded when the mycelium had completely overgrown the control disc. This took from 3 to 11 days depending on the pathogen under culture. The total effect was classified as retarding, inhibiting or promoting the growth.

3. Results

3.1. Method optimization

Methods for testing the antimicrobial activity of plant extracts and natural substances are us-

Table 2
Effects of leaf extracts and coumarins against plant pathogenic fungi^a

Extracts	Microbial species						
	Coumarins	<i>Botrytis cinerea</i>	<i>Fusarium culmorum</i>	<i>Heterobasidium amosum</i>	<i>Phytophthora (cactorum)</i>	<i>Rhizoctonia solani</i>	<i>Rhizoctonia uni-nucleate strain (264)</i>
<i>A. podagraria</i>	—	—	+	+	!!!	—	+
<i>A. graveolens</i>	—	—	—	+	!!!	—	+
<i>A. archangelica</i>	+	+	+	+	!!!	—	+
<i>L. officinalis</i>	+	+	+	+	!!!	—	+
<i>P. crispum</i>	—	—	—	+	!!!	—	—
<i>P. palustre</i>	+	+	+	+	!!!	+	+
<i>R. graveolens</i>	—	—	+	+	!!!	++	—
Bergapten	+	+	—	!!!	—	—	—
Coumarin	—	—	—	—	—	—	—
Herniarin	—	—	+	—	—	—	+
Scopoletin	!!!	+	+	+	—	+	—
Umbelliferone	—	—	+	—	—	—	—
Xanthotoxin	—	—	+	+	—	—	+
Control (methanol, acetone)	—	—	—	—	—	—	—

^a Sample amount 500 µg ($n = 3-6$). !!!, promotes the growth of the pathogen; —, no effect on the growth of the pathogen; +, retards the growth of the pathogen; ++, inhibits clearly the growth of the pathogen; ++++, inhibits strongly the growth of the pathogen.

usually based on agar-diffusion techniques. In this study, the agar-diffusion method described in the European Pharmacopoeia was further developed to serve as a screening procedure when searching for antimicrobial agents.

The solvent tolerance of the micro-organisms was tested using the following solvents; water, DMSO, methanol and acetone. In order to determine the maximum concentration of DMSO in the samples that would allow the test micro-organisms to reach normal growth, DMSO concentrations of 0, 0.2, 20 and 100% (v/v) were used. Uninhibited growth was evaluated as no toxic effects of the solvent.

Surprisingly, a concentration of 20% DMSO was rather toxic to some of the micro-organisms. On the other hand, a DMSO concentration of 0.2% or pure water only partially dissolved the tested extracts and compounds. Therefore the mixture of 40 volumes of methanol R and 60 volumes of 0.1 M phosphate buffer solution pH 8.0 R used in the European Pharmacopoeia was replaced by methanol or acetone owing to the type of plant extracts and compounds examined. Methanol and acetone are good solvents for the plant extracts and coumarin compounds (used successfully also in earlier investigations, Härmälä, 1991; Eloff, 1998; Ojala et al., 1999) and they did not differ significantly from the buffer solution with respect to antibacterial properties. However, they proved to be too toxic for the plant pathogenic fungi when used in holes or cylinders. In the plant pathogen tests the samples were therefore absorbed in two parts on standardized filter paper discs, which were then left to dry properly before application to the dish.

The hole-plate diffusion method was chosen for bacteria, yeasts and mold, because the large volume of the hole permits lower concentrations to be used. This is advantageous with natural products due to their low solubility. A comparison between the holes and cylinders gave similar results, further supporting the use of holes. The disc diffusion method was used for the plant pathogenic fungi, as stated earlier, due to the solvent toxicity. Reasonably high concentrations can be applied to standard-sized

discs but, if the compound to be examined is not very soluble or active, large volumes have to be used and this method becomes relatively slow and laborious (Brantner et al., 1994).

3.2. Antimicrobial effects of leaf extracts

The diameters of the inhibition zones obtained with the leaf extracts are presented in Tables 1 and 2. Screening of the antimicrobial activity of the extracts revealed only slight activity against bacteria, yeasts and mold. *P. crispum* and *R. graveolens* affected the widest spectrum of microbes. On the other hand, most of the leaf extracts inhibited the growth of the plant pathogenic fungi *F. culmorum*, the uninucleate strain of *Rhizoctonia* and all of them inhibited *H. annosum*, while the growth of *Phytophthora (cactorum)* increased in the presence of these extracts. *P. palustre* exhibited activity against the widest spectrum of plant pathogenic fungi. The most significant activity was observed with *R. graveolens*, which inhibited the growth of *R. solani*. The measurements indicated that the largest zones of inhibition were obtained with this leaf extract against all the microbes tested. An HPLC method was applied to determine the amounts of coumarins in the extracts (Vuorela et al., 1989). The average total coumarin contents in all the plants are shown in Table 1.

3.3. Antimicrobial activity of coumarins

The antimicrobial activity of coumarins, which are common constituents in the above species, is shown in Tables 1 and 2 as inhibition zones. The pure coumarins exhibited a very modest activity against the bacteria, yeasts and the mold. Bergapten slightly retarded the growth of *Botrytis*. Scopoletin, umbelliferone, xanthotoxin, and herniarin exhibited strong inhibition of the growth of *F. culmorum*. In contrast, scopoletin promoted the growth of *B. cinerea*, and bergapten of *Heterobasidion annosum*. *Fusarium* proved to be the most sensitive of the pathogenic fungi.

4. Discussion and conclusions

These results suggest the presence of either good antimicrobial potency of the extract or of a high concentration of an active principle in the extracts of *P. crispum* and *R. graveolens*. It should be supposed that essential oils or flavonoids might also be responsible for this activity. This is supported by the overall poor activity shown by the pure coumarins, although in earlier studies some antibacterial inhibitory effects have been found for xanthotoxin, herniarin, umbelliferone and scopoletin, and antifungal activity for umbelliferone, scopoletin, and coumarin (e.g. Jurd et al., 1971a,b; Recio et al., 1989; Kayser and Kolodziej, 1997; Kwon et al., 1997). Substances could also be present in the extract that stimulate the growth of the microorganisms, as was especially evident in the case of *Phytophthora (cactorum)*, thus counteracting the effect of inhibitory substances.

The selected plant species had total coumarin contents of between 5.0 and 834 µg/ml in their methanolic leaf extracts and, as they were merely active against plant pathogens, this study supports the role of coumarins and furanocoumarins as defensive compounds. The method used in this study is discriminatory, for e.g. bioassay-directed isolation of active components. The data presented here justify the inclusion of leaf extracts of *P. crispum* and *R. graveolens* in further investigations on their antimicrobial properties.

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