Tentative Identification of Polyphenols in Sempervivum tectorum and Assessment of the Antimicrobial Activity of Sempervivum L.

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Tentative Identification of Polyphenols in *Sempervivum tectorum* and Assessment of the Antimicrobial Activity of *Sempervivum* L.

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Polyphenols were isolated from sliced fresh leaves of *Sempervivum tectorum*. After 21 h of extraction by methanol and removal of chlorophyll, ethyl acetate was used to separate oligomeric and polymeric polyphenols: 0.07% of oligomeric and 0.13% of polymeric polyphenols were found. After acidic hydrolysis of the oligomeric polyphenols, it was established by TLC, HPLC, and FAB mass spectra that kaempferol was the unique aglycon of the three main oligomeric constituents of *S. tectorum*. Paper chromatography suggested delphinidol to be the only anthocyanidin detectable in the material obtained by acidic hydrolysis of the polymeric polyphenol fraction. After Haslam degradation of the same polymeric polyphenol fraction, only 4-thiobenzyl-(−)-epigallocatechin and 4-thiobenzyl-(−)-epigallocatechin-3-gallate were found and tentatively identified. We concluded that procyanidins of B2 type could be the major components of the polymeric polyphenol fraction of this plant. Antimicrobial activity of *Sempervivum* L. leaves against six of seven selected microorganisms was observed.

Keywords: *Sempervivum tectorum; oligomeric polyphenols; polymeric polyphenols; plant phenolics; antimicrobial effect*

INTRODUCTION

There is considerable interest in the possible use of natural antimicrobials from plants as food additives either to prevent the growth of food-borne pathogens or to delay the onset of spoilage. Many naturally occurring compounds such as polyphenols and tannins have been considered in this context, although polyphenols in foods serve purposes other than as antimicrobial agents (Nychas, 1995).

Herbs and spices are known to be able to prevent or delay the growth of microorganisms (Schmitz et al., 1993; Nychas, 1995; Takenaka et al., 1997), and many researchers consider plant phenolic compounds as those responsible for antimicrobial activity (Haslam, 1989; Nychas et al., 1990; Pathak et al., 1991; Tranter et al., 1993; Tassou and Nychas, 1994).

Polyphenols are secondary metabolites present in all plants and are involved in the development of color, taste, and palatability, as well as the defense system of plants (Sastry and Sastry, 1993; Tarnai et al., 1994). Nearly all phenols are formed either by condensation of C_2 units from acetyl-CoA or through shikimic acid to C_6-C_2 derivatives (Swanson, 1993; Sastry and Sastry, 1993; Strack, 1997). Both biosynthetic pathways are complicated, with many steps, through which, by control of the presence and activity of certain enzymes in a plant, different phenolic compounds are formed (Lee, 1992).

Sempervivum tectorum (syn. *Sempervivum glaucum* Tenore, *S. alpinum*, *S. murale*, *S. schottii*) is an evergreen plant belonging to a large family of the Crassulaceae (Hegnauer, 1964; Thomas and Andre, 1987) with crassulacean acid metabolism (CAM) (Pilon-Smits et al., 1996). There are ~1200 varieties of *Sempervivum*. They can be found in arid areas from 200 to 2800 m above sea level, spread through almost all Mediterranean countries from Spain to Asia Minor. The succulent leaves of *S. tectorum* are conical and juicy, do not smell, have a rather sour taste, and are astringent. In summer the plant develops 10−30 cm long stalks and blossoms in July and August (Swart, 1991).

Fresh juice from squeezed leaves of *S. tectorum* is used as a folk medicine almost exclusively for external purposes. It can be spread as a pack on wounds, sores, burns, and abscesses and also on painful areas attacked by gout as a refrigerant and astringent. Drinking tea prepared from leaves of *S. tectorum* is recommended for ulcer treatment (Bremness, 1996). The fresh juice has long been used as a sure remedy for insect bites. It also eases ear pains and inflammations and helps in easier removal of corns and freckles. In alpine regions *S. tectorum* has been used also in ointments for wounds and other injuries in veterinary practice (Gessner, 1974). Recently, it was found that *S. tectorum* extract has a lipid-lowering effect in rats (Blazovics et al., 1993, 1994) and antioxidative properties (Kerry et al., 1992).

Here, we report the extraction and tentative identification of polyphenols from *S. tectorum* and the results of tests for inhibition of growth of seven microorganisms by homogenized fresh leaves of *Sempervivum* L.

MATERIALS AND METHODS

Reagents. All reagents used were of analytical or HPLC grade purity.

Plant Material. *S. tectorum* plants were from the Botanical Garden in Ljubljana, whereas *Sempervivum* L. for antimicrobial activity tests were collected in the field and used immediately. The plant leaves were washed, excess water was removed, and leaves were then used immediately.

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Extraction of Polyphenols. Extraction procedures for phenolic compounds were performed following the methods of Tarnai et al. (1994). Polyphenols were isolated from 90 g of fresh, clean leaves of *S. tectorum*. The leaves were sliced directly into 900 mL of methanol. After 4 h of extraction during constant stirring at room temperature, the first filtrate was obtained after filtration. To the remaining plant material was added 450 mL of fresh methanol. During constant stirring, a second extraction was run for 17 h at room temperature. Again, the extract was filtered and the second filtrate was combined with the first. The total volume of combined filtrates was ~1400 mL. Methanol was evaporated from the combined filtrates in a vacuum at 30–40 °C so that ~500 mL of the solution remained. The residual solution was diluted with water to 1:1 (v/v) and transferred to a separating funnel where chlorophyll was removed with 150 mL of CH_2Cl_2. The separation of phases was improved by adding ~5 g of NaCl. When the phases were well separated, the organic phase was removed and discarded. The water phase was transferred to a rotavapor. Methanol was evaporated off and the residue diluted to 250 mL. The solution was transferred to a separating funnel. After extraction with 3 × 100 mL of ethyl acetate, two phases were obtained: the *ethyl acetate phase*, presumed to contain ethyl acetate soluble oligomeric polyphenols, and the remaining *water phase*, expected to contain ethyl acetate insoluble polymeric polyphenols.

The ethyl acetate phase was first dried over anhydrous Na_2SO_4, ethyl acetate was evaporated off, and the residue was dried under a stream of nitrogen. The dried residue was treated with 5 mL of CH_2Cl_2 to dissolve nonphenolic constituents, and the insoluble fraction was recovered after filtration, dried, and weighed. Some of the dry residue was used for acidic hydrolysis; the remainder was peracetylated.

Acidic Hydrolysis of Oligomeric Glycosides. Glycosides were hydrolyzed by ethanol/2 N HCl (1:1 v/v), for 1 h at 100 °C (Harborne, 1965).

Peracetylation. To 60.2 mg of the insoluble fraction were added 100 μL of pyridine and 500 μL of acetic anhydride. After 6 h at room temperature in the dark, the reaction mixture was poured into ice–water, where peracetylated derivatives precipitated (Piretti and Doghieri, 1990). The precipitate was filtered, dried over $CaSO_4$, and used for TLC and HPLC analysis.

The water phase was purified by ion exchange chromatography using Amberlite XAD-4 (Rhom-Haas Co., Milano, Italy) in a glass column (2 × 10 cm); polymeric polyphenols were eluted with 190 mL of methanol. The solvent was evaporated off and the residue dried under a stream of nitrogen (Tarnai et al., 1994). Some portion (milligrams) of the obtained residue was used for qualitative analysis. After this was dissolved in ethanol, a few drops of concentrated HCl was added and the solution was boiled until the red color reached maximum intensity (Bate-Smith, 1954). Anthocyanidins formed after acidic hydrolysis were checked by paper chromatography using Forestal mixture [CH_3COOH (concentrated)/H_2O/HCl (concentrated) (30:10:3 v/v)] and Whatman No. 1 chromatography paper (Markham, 1975).

Benzylmercaptan Cleavage of Polymeric Polyphenols. The dried residue (113.5 mg), which presumably contained polymeric polyphenols, was dissolved in 20 mL of 95% C_2H_5OH, and, following the Haslam degradation procedure, 2 mL of concentrated CH_3COOH and 2 mL of benzylmercaptan were added (Thompson et al., 1972). The mixture was refluxed for 48 h at 110 °C under a constant stream of nitrogen through the solution. After benzylmercaptan cleavage and evaporation of C_2H_5OH, the obtained 4-thiobenzylated compounds appeared as red oily products. The products were stuck to the flask well separated from the colorless liquid mixture, so the latter could be easily removed by decantation. The oily residue was peracetylated (Piretti and Doghieri, 1990), and the peracetylated products were recovered according to the method of Tarnai et al. (1994) and analyzed by HPLC.

Qualitative Analysis. Peracetylated derivatives were analyzed by TLC on silica gel 60 plates in the dark with benzene/acetone (8:2 v/v) as mobile phase. The spots were detected at 150 °C after the plates were sprayed with H_2SO_4/HCHO (40%) (9:1 v/v) (Tarnai et al., 1994).

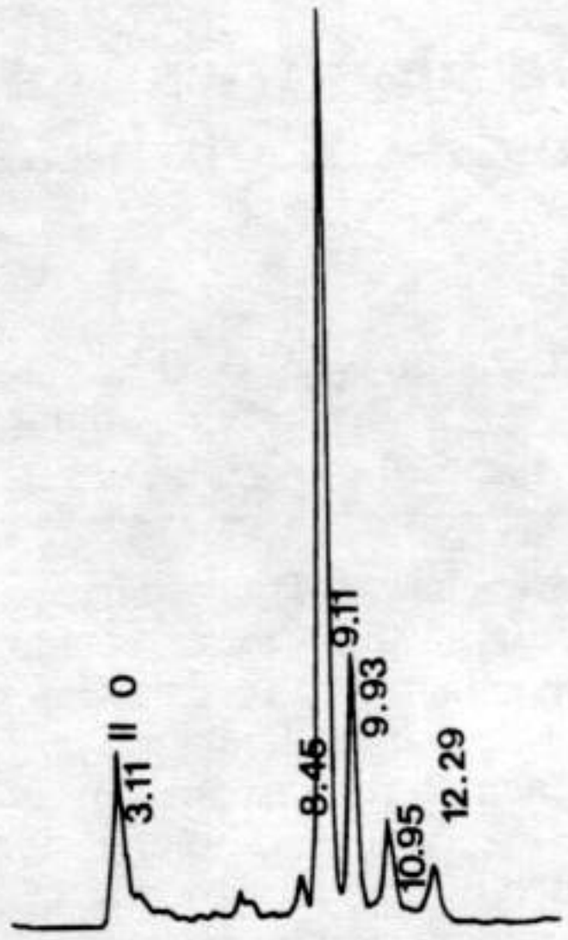

Figure 1. HPLC profile of peracetylated oligomeric polyphenols isolated from leaves of *S. tectorum* on a Spherisorb S-5 nitrile column (250 × 4.6 mm). Isocratic elution was performed by *n*-hexane/ethyl acetate (6:4 v/v) at a flow rate of 1 mL/min with detection at 278 nm.

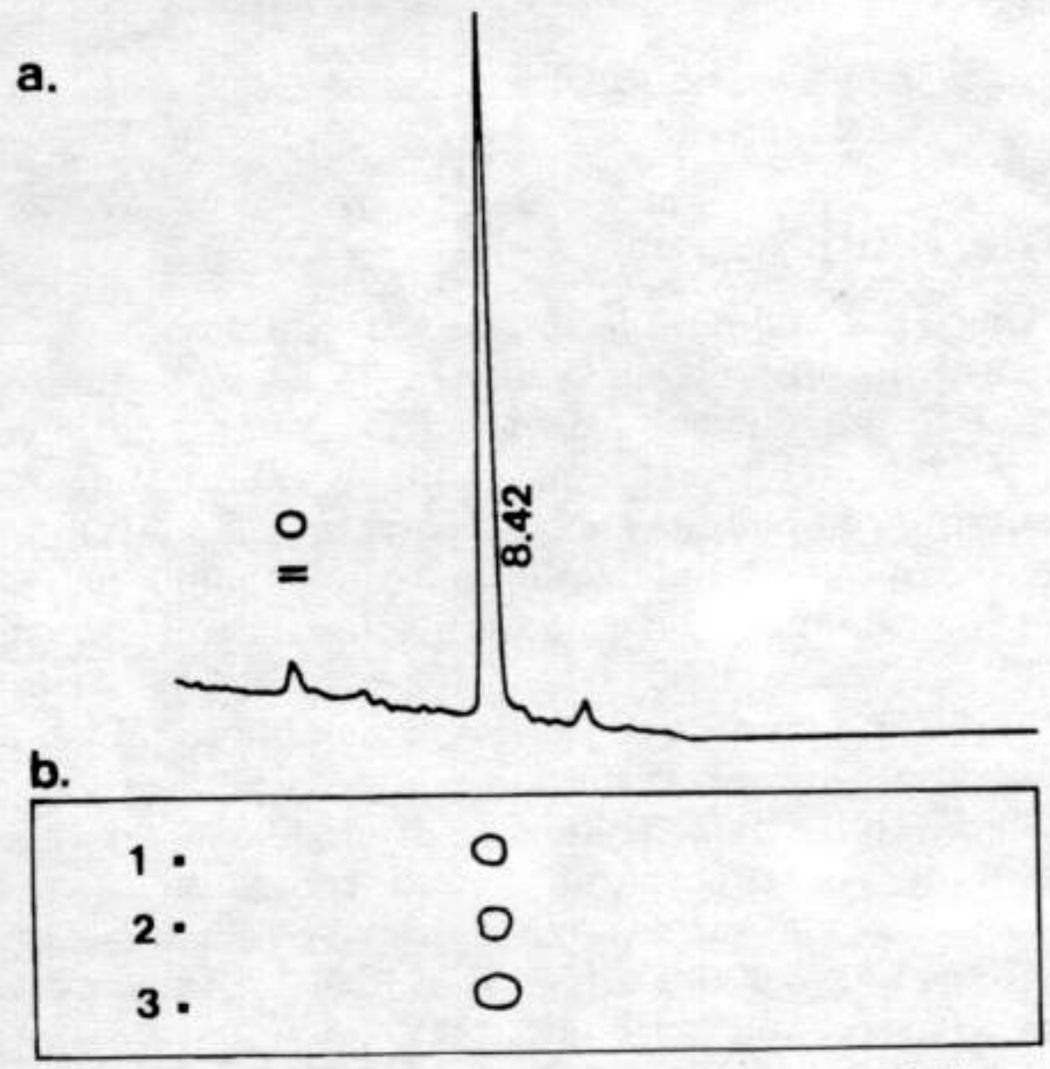

Figure 2. (a) HPLC of peracetylated aglycon of oligomeric polyphenols isolated from the leaves of *S. tectorum* on a Spherisorb S-5 nitrile column (250 × 4.6 mm). The HPLC conditions were the same as in Figure 1. (b) TLC of peracetylated aglycon of oligomeric polyphenols (**1**) and a 1:1 (v/v) mixture of peracetylated aglycon of oligomeric polyphenols and kaempferol (**2**) and (**3**).

HPLC of Peracetylated Derivatives. A 5 μm Spherisorb S-5 nitrile column (250 × 4.6 mm, Phase Separations, Queensferry, U.K.) coupled with a precolumn (100 × 4.6 mm) packed with Partisil-10 PAC (Whatman, Clifton, NJ) was used. Peaks were monitored at 278 nm. Isocratic elution with *n*-hexane and ethyl acetate (6:4 v/v) was carried out at a flow rate of 1 mL/min (Piretti et al., 1994). Ten microliters of the sample was injected after such dilution that the peak height was ~50% of the whole range.

Mass Spectrometry. FAB mass spectra of the corresponding protonated or deprotonated molecules of kaempferol and kaempferol glycosides were obtained on an AutoSpecQ mass spectrometer (Micromass, Manchester, U.K.), using Cs^+ ion bombardment (30 keV) ionization and *m*-nitrobenzyl alcohol as the matrix.

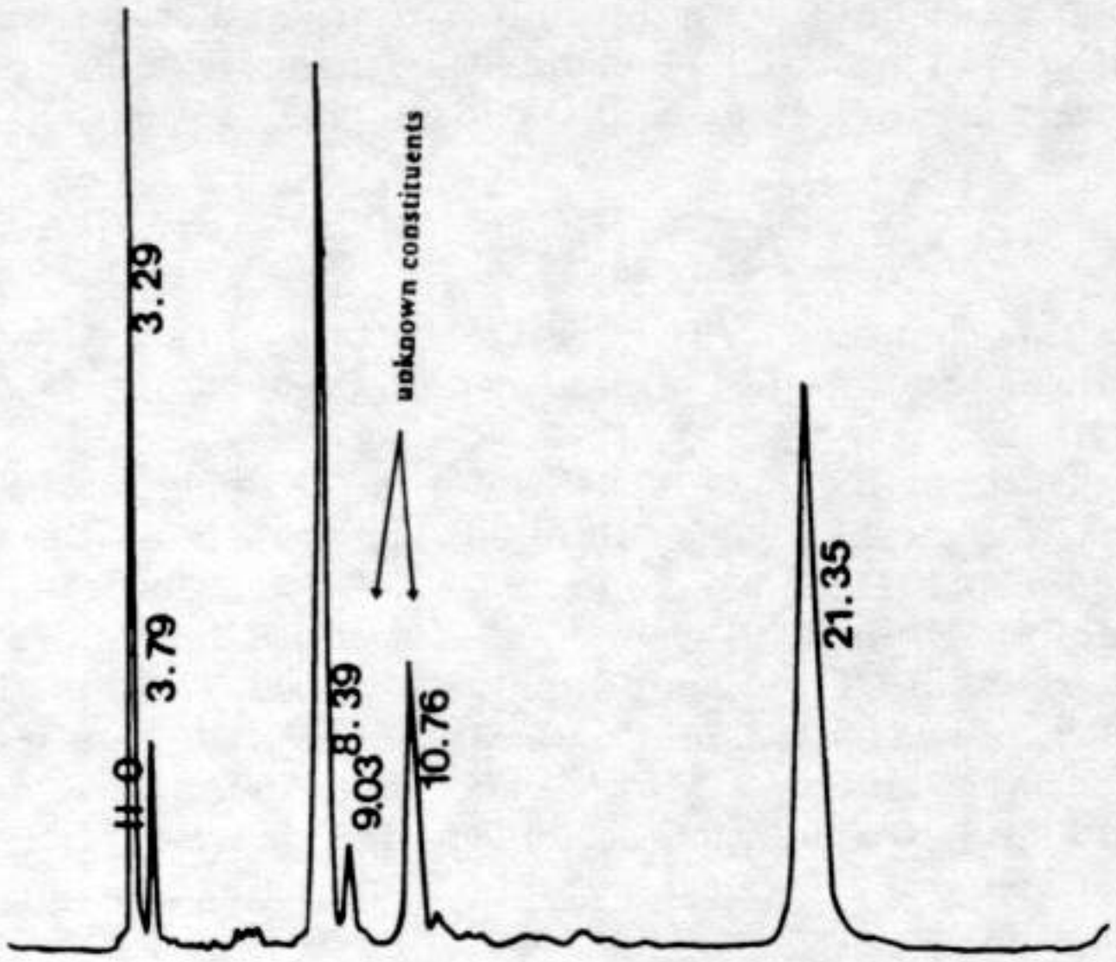

Figure 3. HPLC profile of peracetylated 4-thiobenzyl derivatives of polymeric polyphenols on a Spherisorb S-5 nitrile column (250 × 4.6 mm). The HPLC conditions were the same as in Figure 1. Isocratic elution was performed by *n*-hexane/ethyl acetate (6:4 v/v) at a flow rate of 1 mL/min with detection at 278 nm.

Microorganisms. Seven microorganisms were selected to test the antimicrobial activity of *Sempervivum* L.: *Bacillus cereus* (isolated from water), *Proteus morgani*, *Staphylococcus aureus* (isolated from fermented sausage), *Enterococcus faecalis* (isolated from a cheese product), *Escherichia coli*, *Saccharomyces cerevisiae*, and *Geotrichum* sp. (isolated from cabbage). Selected microorganisms were from either the Cultural Collection of the Biotechnical Faculty or the Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana. Cultures of bacteria were maintained in 15% glycerol at −18 °C. Yeast was grown on malt extract agar (Oxoid CM 59) and mold on Sabouraud dextrose agar slants (Oxoid CM 41) at 4 °C. Overnight cultures of bacteria were prepared in nutrient broth No. 2 (Oxoid CM 67) and overnight cultures of yeast and mold in Sabouraud broth (Biolife 402000) before inoculation.

Antimicrobial Tests. For cultures of bacteria, nutrient broth No. 2 (Oxoid CM 67) was used and for the yeast and mold a Sabouraud broth (Biolife 402000). To 95 or 90 mL of broth in an Erlenmeyer flask was added 5 or 10 g of clean, fresh leaves of *Sempervivum* L., and the mixture was homogenized at 4 °C with an Ultraturrax at maximum speed for 2 × 1 min. The homogenate was then inoculated with the selected microorganism and incubated at an appropriate temperature. After 1, 2, 3, and 5 h of incubation, 1 mL of broth was taken from each flask and diluted to 10 mL with saline solution. Different dilutions of broth were prepared by consecutive dilutions until a maximum number of 300 CFU per plate was obtained, 0.1 mL of diluted broth being always the volume spread on each plate prepared with growth medium: *B. cereus*, Bacillus Cereus Agar Base (Oxoid CM 617); *P. morgani*, Brilliant Green Agar (Oxoid CM 263); *S. aureus*, Baird-Parker medium (Oxoid CM 275); *E. faecalis*, Slanetz-Bartley Agar (Oxoid CM 377); *E. coli*, Eosin Methylene Blue (Oxoid CM 69); *S. cerevisiae* and *Geotrichum* sp., OGY Agar (Biolife 1838). Agar plates were incubated for 24 or 48 h at the optimal temperature for each microorganism. All dilutions were performed in three replicates.

RESULTS AND DISCUSSION

Using the Bate-Smith method (Bate-Smith, 1973; Bate-Smith and Rašper, 1969) for qualitative determi-

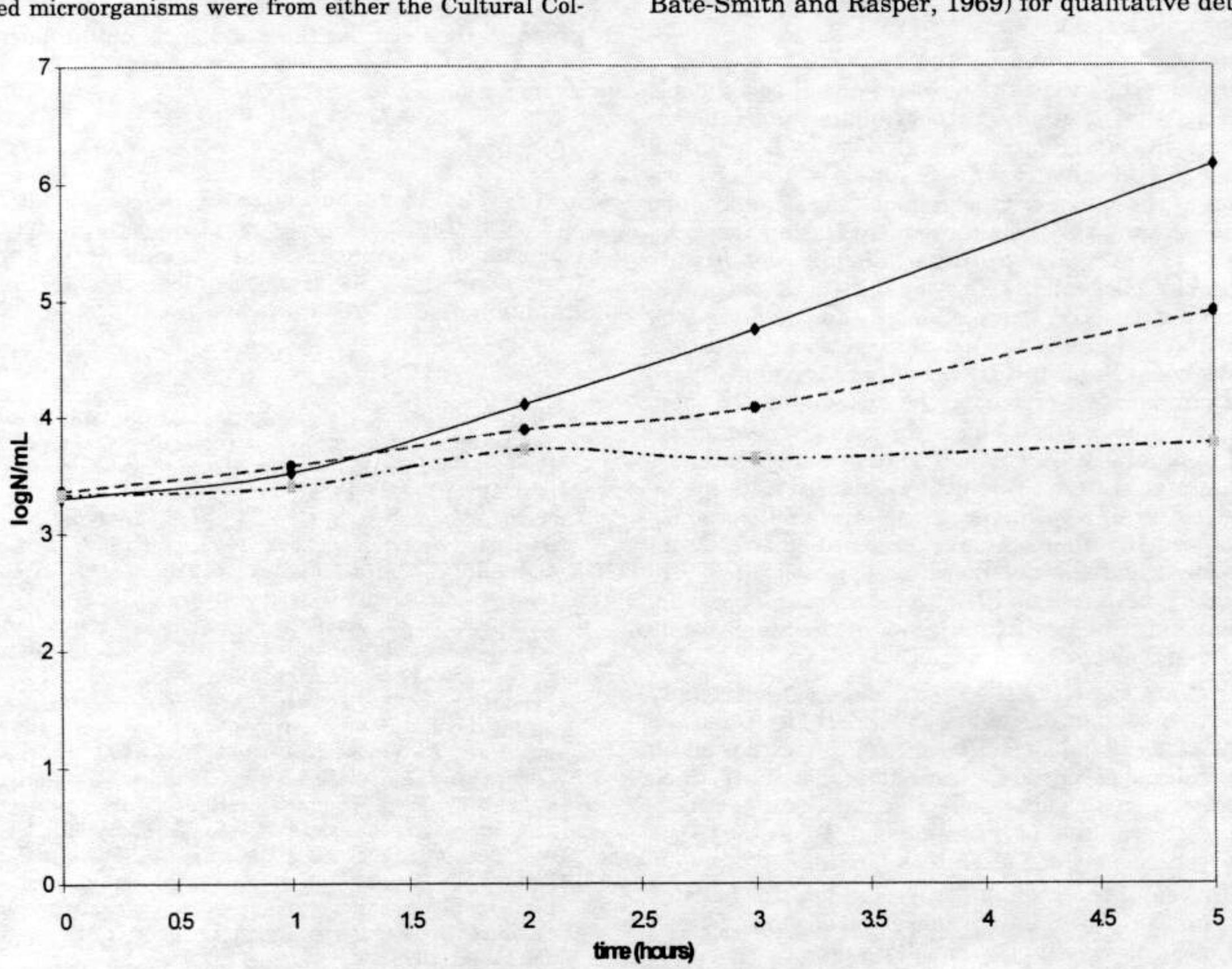

Figure 4. Kinetics of growth of *S. aureus* after incubation with homogenized fresh leaves of *Sempervivum* L.: 5% (- - -) and 10% (- · · -) (w/v) addition; (—) control.

nation of phenolic compounds and from the absorbance at 538 nm, we previously estimated that there was ~3 times more phenolic compounds present in the skin of the leaves than in the juice itself (Abram et al., 1995). The content of total phenolics in juice was then determined according to the Folin–Denis method with Folin–Ciocalteu reagent (Amerine and Ough, 1973): 1.414 mg of total phenolics/mL of juice from fresh leaves was found. Following the above extraction procedures, we isolated 0.07% of oligomeric and 0.13% of polymeric polyphenols from fresh leaves of *S. tectorum*. After peracetylation of oligomeric polyphenols, three yellow spots were visible on the TLC plate, suggesting that the three main oligomeric constituents could be flavone derivatives, possibly flavone glycosides. The HPLC chromatogram in Figure 1 shows the presence of five peaks with retention times of 8.45, 9.11, 9.93, 10.95, and 12.29 min. After acidic hydrolysis of the oligomeric polyphenols, subsequent recovery, and peracetylation of the aglycon moieties, using TLC with silica gel 60, we detected only one yellow spot with the same R_f as the peracetylated kaempferol standard (Figure 2b). The HPLC chromatogram (Figure 2a) suggested that a single aglycon, having a retention behavior matching that of kaempferol (3,4′,5,7-tetrahydroflavone), was found in the oligomeric polyphenolic fraction of this plant. FAB mass spectra of the protonated molecules of kaempferol showed the presence of ions with *m/z* 287 (MH^+), and mass spectra of deprotonated kaempferol glycoside showed ions with *m/z* 447 $(M - H)^+$. The results of these analyses therefore confirmed the presence of kaempferol as the only aglycon in this oligomeric polyphenolic fraction.

Paper chromatography of the products formed after the Bate-Smith reaction with the polymeric polyphenols suggested that the only anthocyanidin detectable in the material after acidic hydrolysis was delphinidol (3,3′,4′,5,5′,7-hexahydroxyflavilium ion). Further purification of the polymeric polyphenols by ion exchange chromatography and their subsequent Haslam degradation was also carried out to determine the constituents of the proanthocyanidins. The products of this degradation were recovered, peracetylated, and analyzed by HPLC under the conditions described under Materials and Methods. From the HPLC chromatogram (Figure 3) and standards we presumed the presence of 4-thiobenzyl-(−)-epigallocatechin and 4-thiobenzyl-(−)-epigallocatechin-3-gallate. 4-Thiobenzylated (−)-epigallocatechin eluted after 8.39 and 4-thiobenzylated (−)-epigallocatechin-3-gallate at 21.35 min; the two peaks at 9.03 and 10.06 min remain unidentified. We tentatively identified the polymeric polyphenols in *S. tectorum* as procyanidins of B2 type, where (−)-epigallocatechin and (−)-epigallocatechin-3-gallate are probably linked together by C_4–C_8 bonds.

As Pathak et al. (1991) report, (−)-epigallocatechin and similar compounds with 3′,4′,5′ -OH groups on the aromatic ring B and an −OH group at the third position are considered essential for antibacterial activity. Such compounds can also act as effective repellents to predators, whether animal or antimicrobial, according to the earlier conclusions of Bate-Smith (1973).

Inhibition of the growth of selected microorganisms in the presence of 5% addition of homogenized fresh leaves of *Sempervivum* L. in media was evaluated after 5 h of incubation. The results were expressed as the number of colony-forming units (CFU) grown on agar plates per milliliter of broth (CFU/mL) and calculated as growth inhibition percentages (I) using the following formula (Rico-Mûnoz and Davidson, 1983):

$$I = (C - T/C) \times 100$$

In this equation, C = CFU/mL of control and T = CFU/mL in the presence of homogenated *Sempervivum* L. leaves.

The growth of *S. aureus* (94.5%) and *B. cereus* (92.1%) was the most strongly inhibited. The growth of *Geotrichum* sp. (60.0%) and *E. faecalis* (57.5%) was moderately inhibited, and the growth of *E. coli* (9.3%) and *P. morgani* (5.1%) was only slightly inhibited. The growth of *S. cerevisiae* was not noticeably inhibited. Figure 4 shows the kinetics of growth of *S. aureus* in the presence of 5 and 10% addition of homogenized leaves of *Sempervivum* L. after 1, 2, 3, and 5 h of incubation when an aliquot of broth was taken, diluted by 10^3 or 10^4, and spread on agar plates prepared with Baird–Parker medium (Oxoid CM 275).

In our experiments *S. aureus* showed the most growth inhibition in the presence of leaves of *Sempervivum* L. According to Nychas (1995), phenolic compounds could be held responsible for such action on microorganisms, either because they inhibit the enzymes necessary for the production of energy in cells or because they cause changes in cell membrane permeability or inhibit lipoxygenase and cyclooxygenase and, as suggested in the case of *S. aureus* (Pathak et al., 1991), can influence RNA synthesis. Our future aim is to isolate polyphenolic components from leaves of *Sempervivum* L. plants grown in the field, test their ability to inhibit the growth of *S. aureus*, and research the potential for their use as antimicrobials.

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