ORIGINAL ARTICLE

Antibacterial activity of sphagnum acid and other phenolic compounds found in *Sphagnum papillosum* against food-borne bacteria

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Abstract

Aims: To identify the phenolic compounds in the leaves of *Sphagnum papillosum* and examine their antibacterial activity at pH appropriate for the undissociated forms.

Methods and Results: Bacterial counts of overnight cultures showed that whilst growth of *Staphylococcus aureus* 50084 was impaired in the presence of milled leaves, the phenol-free fraction of holocellulose of *S. papillosum* had no bacteriostatic effect. Liquid chromatography–mass spectrometry analysis of an acetone–methanol extract of the leaves detected eight phenolic compounds. Antibacterial activity of the four dominating phenols specific to *Sphagnum* leaves, when assessed *in vitro* as minimal inhibitory concentrations (MICs), were generally >2·5 mg ml^{-1}. MIC values of the *Sphagnum*-specific compound 'sphagnum acid' [*p*-hydroxy-β-(carboxymethyl)-cinnamic acid] were >5 mg ml^{-1}. No synergistic or antagonistic effects of the four dominating phenols were detected in plate assays.

Conclusions: *Sphagnum*-derived phenolics exhibit antibacterial activity *in vitro* only at concentrations far in excess of those found in the leaves.

Significance and Impact of the Study: We have both identified the phenolic compounds in *S. papillosum* and assessed their antibacterial activity. Our data indicate that phenolic compounds in isolation are not potent antibacterial agents and we question their potency against food-borne pathogens.

Introduction

The bog moss *Sphagnum papillosum* is the dominant peat former in acid peatlands in northern Europe (Daniels and Eddy 1985) and is considered to be responsible for the preservative action on intact human remains ('bog-bodies') (Daniels and Eddy 1985; Turner 1995; Fischer 1999). Sphagnum acid (SA) is a phenolic compound specific to *Sphagnum* species (Rudolph and Samland 1985; Rasmussen *et al.* 1995; van der Heijden *et al.* 1997; Williams *et al.* 1998) which has been implicated in the preservative action of the moss since breakdown products of SA have been identified in bog-bodies (Stankiewicz *et al.* 1997). Correspondingly, phenolic compounds of different moss and plant species have been shown to possess antibacterial activity (Fern-

àndez *et al.* 1996; Basile *et al.* 1999; Rauha *et al.* 2000; Friedman *et al.* 2003) as do plant extracts (Fernàndez *et al.* 1996; Basile *et al.* 1998; Rauha *et al.* 2000; Singh *et al.* 2007), but there are no studies on the antibacterial effect of SA.

The proposed action of plant phenolics in tissue preservation may have application to the food industry. Thus, we examined the effect of *S. papillosum* and a chlorite-treated fraction, free of aromatic compounds (holocellulose) against recognized food-borne pathogens. The contribution of the phenolic components identified in *S. papillosum* were examined using *in vitro* antibacterial assays. Since undissociated forms of weak organic acids are the forms that exert antibacterial action (Lund and Eklund 2000), we have carried out the assays at a pH that yields the greatest proportion of undissociated acid

compatible with bacterial growth in an minimal inhibitory concentration (MIC) assay (pH 5).

Materials and methods

Preparation of leaves and holocellulose of *S. papillosum*

The *Sphagnum* plants used in this study were from the same batch as used and described by Ballance *et al.* (2007). *Sphagnum papillosum* plants were collected in August 2003 in Tømmerdalen, Trondheim, Norway. The plants were treated as described previously (Ballance *et al.* 2007), where dried leaves were boiled in acetone and chlorine dioxide was used to selectively oxidize aromatic compounds. Chlorite-treated leaves are referred to as holocellulose.

Bacterial strains

Staphylococcus aureus 50084 (National Veterinary Institute), *Bacillus cereus* NVH 0075/95 (Norwegian School of Veterinary Science), *Salmonella* Typhimurium E224/87 (Norwegian Institute of Public Health) and *Shewanella putrefaciens* ATCC 8071 were used as test organisms in this study, all originally isolated from contaminated food.

Phenolic compounds

The following 11 phenolic compounds were detected by cupric oxide (CuO) oxidation of several *Sphagnum* species by Williams *et al.* (1998) and used as standards for liquid chromatography–mass spectrometry (LC–MS) analysis in our study: *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, SA [*p*-hydroxy-*β*-(carboxymethyl)-cinnamic acid], *p*-hydroxybenzaldehyde, vanillin (4-hydroxy-3-methoxybenzaldehyde), *p*-coumaric acid (4-hydroxycinnamic acid), acetovanillone (4-hydroxy-3-methoxyacetophenone), syringaldehyde (4-hydroxy-3,5-dimethoxy

benzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid) and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid). All chemicals and solvents were of analytical and high-performance liquid chromatography grade. All standards except SA were supplied by Sigma-Aldrich Norway AS. SA was purchased from Exclusive Chemistry Ltd (Russia) and confirmed by measuring the molecular weight using LC–MS.

Chromatographic conditions and sample pretreatment

A Sciex API 2000 mass spectrometer equipped with an electrospray interface (Applied Biosystems, Ontario, Canada) was operated in a negative electrospray mode quantitation transition (Table 1). The analytical (PLRP-S, 5 μm, 150 × 4·6 mm i.d.) and guard columns (5 × 3 mm i.d.) (Polymer Laboratories, Amherst, MA, USA) were operated at a constant temperature of 27°C. The mobile phase consisted of a mixture of solution A (980 ml water + 20 ml methanol + 0·1 ml NH_3 (25% solution)) and solution B (methanol only). The pump programme operated conditions were: 1·5 min 100% solution A, 3·7 min 10% solution A and 90% solution B with a flow rate of 0·8 ml min^{-1}. This was followed by 8·8 min 100% solution A with a flow rate of 1 ml min^{-1} and finally 1 min 100% solution A with a flow rate of 0·8 ml min^{-1}. The LC fluent was split approx. 1:4 before entering the MS interface. Volumes of 0·1 ml NH_3 (25% solution) and 2850 ml acetone–methanol (6 + 4) were added to 50 mg dried *Sphagnum* moss (milled leaves), whirl-mixed for 6 s and left in an ultrasonic bath for 15 min. The sample was shaken for 3 s and then centrifuged for 3 min (3250 *g*). Supernatant and water (1 + 2 v/v) was mixed thoroughly and filtered through a Spin-X centrifuge filter. Aliquots of 30 μl were injected into the LC–MS/MS at intervals of 15 min for the determination of *Sphagnum* moss compounds. All the 11 phenolic compounds were included

Phenolic compound	Mw M⁻	Fragment ion	Fragment ion	Limit of quantification (μg g^{-1})	Limit of detection (μg g^{-1})
Vanillic acid	167·2	91·1	108	1·0	0·5
p-hydroxybenzoic acid	137·2	93·1	65·1	1·0	0·5
Syringic acid	197	121·1	89	0·5	0·3
p-coumaric acid	163	119	93·1	1·0	0·5
ferulic acid	193·2	134	89·1	0·7	0·3
p-hydroxy-benzaldehyde	121·1	92·1	65·1	0·2	0·1
Vanillin	151·1	136·1	92	0·3	0·1
Syringaldehyde	181·2	166	151	0·2	0·1
p-hydroxy-acetophenone	135·2	92·1	120	0·2	0·1
Acetovanillon	165·2	150	122·1	0·3	0·1
Sphagnum acid	221·1	177	133·1	1·0	0·5

Table 1 Phenolic compound used as standards in LC-MS/MS. Fragment ion with slow scrip was used for confirmation

once in each experiment, and each experiment was repeated ten times ($n = 10$).

Bactericidal assays

Milled leaves and holocellulose

Antibacterial activity of suspensions of ground leaves of *S. papillosum* and holocellulose were evaluated against *Staph. aureus* 50084. A total of 20, 10 and 5 mg ml^{-1} suspensions of milled moss and 20 and 10 mg ml^{-1} suspensions of holocellulose were made in tryptic soy broth (TSB; Difco, Sparks, MD, USA) containing 0·1 mol l^{-1} 4-morpholinoethanesulphonic acid (MES) pH 7 and autoclaved at 121°C for 15 min. Once cooled to room temperature overnight suspensions of *Staph. aureus* ($1·4 \times 10^4$–$3·1 \times 10^4$ CFU ml^{-1}) grown in TSB 0·1 mol l^{-1} MES pH 7 were added and incubated at 37°C overnight at 100 rev min^{-1}. Visible turbidity after 18 ± 1–2 h was recorded and the number of colony forming units (CFU) on blood agar plates incubated at 37°C overnight from the different suspensions of the moss were compared with the number of CFU from the growth control of the strain to assess the antistaphylococcal activity of the moss. The pH in the different suspensions were measured both before and after conducting the experiment (Porotrode, Metrohm, Norway) and appropriate growth controls of the strain at lower pH were included when considered necessary. Three separate experiments were performed for each condition.

MIC and MBC determinations

About 20 mg ml^{-1} solutions of *p*-hydroxyacetophenone and *p*-hydroxybenzaldehyde were prepared in TSB 0·2 mol l^{-1} MES pH 7 at 90–100°C and filtered (0·2 μm). Five hundred micro litre volumes of serial twofold dilutions of the phenolic compounds were mixed with equal amounts of $5·0 \times 10^4$–$2·4 \times 10^5$ CFU ml^{-1} of overnight cultures of the test organisms (grown in TSB 0·2 mol l^{-1} MES pH 7) and incubated at 37°C for 18 ± 1–2 h. *Staphylococcus aureus* 50084, *Salm.* Typhimurium E224/87 and *B. cereus* NVH 0075/95 were included as test strains. MIC was read as the lowest concentration of the phenolic compounds that inhibited visible bacterial growth. Minimal bactericidal concentration (MBC) assays were performed by plating 10 μl or 100 μl aliquots from the test tubes on blood agar plates and incubated at 37°C for 18 ± 1–2 h. The MBC for each phenol was defined as the lowest concentration reducing the inoculum by >99·0%. The MIC/MBC experiments were repeated twice for each phenolic compound and each bacterial strain ($n = 2$).

About 20 mg ml^{-1} and 10 mg ml^{-1} solutions of *p*-hydroxybenzoic acid and SA respectively, were prepared as described above for *p*-hydroxyacetophenone and

p-hydroxybenzaldehyde in TSB 0·2 mol l^{-1} MES pH 5·5. These two phenolic compounds were tested at around pH 5 in order to obtain the highest proportion of undissociated acid at a pH that sustained bacterial growth. *B. cereus* was not included since pH 5 is the lower limit permitting growth (Lund and Eklund 2000). Serial twofold dilution and addition of a diluted overnight culture of *Salm.* Typhimurium E224/87 and *Staph. aureus* 50084 ($5·0 \times 10^4$–$2·4 \times 10^5$ CFU ml^{-1}) were prepared as for the other two phenolic compounds. The tubes were incubated at 37°C for 18 ± 1–2 h and MIC was recorded and subsequent MBC assays were carried out. The experiments were repeated twice ($n = 2$). Test organisms were acclimatized to growth at pH 5 as appropriate by three successive overnight incubations in TSB containing MES at different pH values. The choice of pH in the test solutions of the phenolic acids was based on the Henderson–Hasselbach equation

$$[HA]\% = 100/(1 + 10^{(pH - pKa)})$$

where, HA is denoted the acid form (undissociated form). The pKa values were obtained using the web-based tool 'pKa DB' (ACD/Labs, Ontario, Canada, ver. 11.01) and found to be 4·6 for the carboxyl group of *p*-hydroxybenzoic acid and 4·8 and 4·1 for the two carboxyl groups of SA.

Diffusion assays

The antimicrobial potential of vanillin, *p*-coumaric acid, acetovanillone and syringaldehyde at 10 mg ml^{-1} in TSB 0·2 mol l^{-1} MES pH 7 were evaluated in a diffusion assay on tryptic soy agar (TSA; Difco). *Staphylococcus aureus* 50084, *B. cereus* NVH 0075/95, *Salm.* Typhimurium E224/87 and *S. putrefaciens* ATCC 8071 were included in this study. A few colonies were transferred from a pure culture of each strain on blood agar plates into 3 ml of peptone water (PW; Oxoid, Basingstoke, UK) to make a turbid solution. 1 ml of PW was then added to the TSA plate, distributed evenly and the excess was removed. Solutions of 10 mg ml^{-1} of the phenols were made by dissolving the phenols in TSB 0·2 mol l^{-1} MES pH 7 (magnetic stirring and heating, 90–100°C) and filtered through 0·2 μm sterile filter. About 100 μl of each solution was added to the TSA plates and left at room temperature for 1 h to let the test compounds prediffuse before incubating the plates at 37°C for 18 ± 1–2 h. The plates inoculated with *S. putrefaciens* ATCC 8071 were incubated at 25°C. Antibacterial activity was judged to be significant if zones of inhibition were observed macroscopically (by eye). In each experiment each phenolic compound was tested in duplicate and the trials were repeated twice ($n = 2$).

Any possible synergistic or antagonistic effects between the four phenolic compounds found in the highest quantities in the leaves of *S. papillosum* (Table 2) were evaluated using a diffusion assay on solid medium (Möller and Holmgren 1969). TSA plates were inoculated with either *Staph. aureus* 50084, *B. cereus* NVH 0075/95, *Salm.* Typhimurium E224/87 or *S. putrefaciens* ATCC 8071 using the same procedure as described in the previous paragraph and left to dry at 37°C for approx. 15 min before the test compounds were added. Paper strips of 0.5×5.0 cm of chromatography paper (Whatman) were soaked in 10 mg ml^{-1} solutions of the phenolic compounds and placed on the agar plate at right angles to one another. TSA plates were preincubated for 1 h at 4°C and then incubated at 37°C for 18 ± 1–2 h before interpreting the growth patterns. Plates inoculated with *S. putrefaciens* ATCC 8071 were incubated at 25°C. The experiments were repeated twice.

Results

Milled leaves and holocellulose

Whilst suspensions of the ground moss of 10 mg ml^{-1} and 5 mg ml^{-1} in TSB pH 7 failed to inhibit the growth

Table 2 Phenolic compounds detected in *Sphagnum papillosum* using LC–MS/MS

Phenolic compound	μg g^{-1} dry weight*	SEM
p-hydroxyacetophenone	60.0	±0.7
p-hydroxybenzoic acid	25.1	±0.5
sphagnum acid	22.4	±0.9
p-hydroxybenzaldehyde	9.1	±0.6
Vanillin	2.2	±0.06
p-coumaric acid	2.0	±0.3
Acetovanillon	0.3	±0.02
syringaldehyde	0.2	±0.008

*The results are given as mean values of 10 separate experiments.

of *Staph. aureus* 50084 (both the moss and control suspensions yielded 10^9 CFU ml^{-1} after 18 h), suspensions of 20 mg ml^{-1} reduced growth by two log-units ($1.3 \pm 0.4 \times 10^7$ CFU ml^{-1} (moss) *vs* $2.5 \pm 0.5 \times 10^9$ CFU ml^{-1} (TSB alone), $P = 0.003$ paired *t*-test, ($n = 3$)). Even though the pH in this suspension decreased to around 6.60–6.80, an additional growth control of the strain showed no reduced growth at this pH compared to pH 7. In the suspensions of 10 mg ml^{-1} and 5 mg ml^{-1} the pH was between 6.80–6.90 and 6.86–6.95, respectively. Suspensions of holocellulose of 20 mg ml^{-1} and 10 mg ml^{-1} had no detectable inhibitory effect on the growth of *Staph. aureus* 50084 ($3.3 \pm 0.6 \times 10^9$ CFU ml^{-1} (holocellulose) *vs* $2.5 \pm 0.5 \times 10^9$ CFU ml^{-1} (TSB alone), $P > 0.05$ paired *t*-test, ($n = 3$)). The pH in the suspensions of 20 mg ml^{-1} was between 6.53–6.77 and in 10 mg ml^{-1} between 6.75 and 6.89.

Contents of phenolic compounds in the leaves of *S. papillosum*

Eight phenolic compounds were detected in milled leaves of *S. papillosum* (see Table 2). The *p*-hydroxyl phenolics including SA accounted for nearly 93% of the total phenolic compounds detected of which *p*-hydroxyacetophenone accounted for approx. 50%.

MIC/MBC assays

The MIC and MBC of the four dominant phenols against the test strains are given in Table 3. Acclimatization of the test strains to pH 5 resulted in comparable numbers of organisms to that obtained in pH 7. The MICs of SA towards *Staph. aureus* 50084 and *Salm.* Typhimurium E224/87 were both higher than the highest test concentration (5 mg ml^{-1}). The MBCs of the three other phenols were found to be higher than the MICs, indicative of a bacteriostatic rather than a bactericidal effect. For

Table 3 Susceptibility testing of three of the test strains. Results are given as the highest obtained MIC and MBC values (in mg ml^{-1})

Phenolic compounds								
	p-hydroxyacetophenone (pH 7)		*p*-hydroxybenzaldehyde (pH 7)		sphagnum acid (pH 5)		*p*-hydroxybenzoic acid (pH 5)	
Bacterial strains	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Staphylococcus aureus 50084	5.0	>10.0	5.0	10.0	>5.0	>5.0	1.25	>10.0
Bacillus cereus NVH 0075/95	5.0	*5.0–>10.0	5.0	*5.0–>10.0	ND	ND	ND	ND
Salmonella Typhimurium E224/87	2.5	>10.0	2.5	10.0	>5.0	>5.0	5.0	>10.0

$n = 2$ in all experiment.

*The results differed with more than one concentration unit (one test tube), therefore the range of the results are given.

ND, not determined; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration.

B. cereus NVH 0075/95 the MBCs of *p*-hydroxyacetophenone and *p*-hydroxybenzaldehyde varied with more than one dilution unit (test tube) between experiments and therefore the range is given.

Diffusion assays

In a disk diffusion assay no inhibition of growth was detected against either *Staph. aureus* 50084, *Salm.* Typhimurium E224/87 or *B. cereus* NVH 0075/95. However, vanillin, acetovanillone and syringaldehyde showed a weak (zones of inhibition <0·5 cm in width) effect towards *S. putrefaciens* ATCC 8071. The fourth phenol tested, *p*-coumaric acid, did not show any inhibition of growth of *S. putrefaciens* ATCC 8071 at pH 7. No synergistic or antagonistic effects between any of the four *p*-hydroxyl phenols were detected on TSA plate assays.

Discussion

The reduction in bacterial numbers following incubation with ground leaf preparations but not the chlorite-treated holocellulose indicate that aromatic compounds are responsible for the antibacterial effect. However, the MIC and diffusion assays results obtained indicate that SA and the other seven phenolic compounds detected in *S. papillosum* do not exert marked antibacterial activity against the bacterial strains tested when tested in isolation.

SA has been suggested to be the phenol responsible for the observed preservative effect of the moss (Verhoeven and Toth 1995) but our results do not support this idea for the following reasons: (i) SA did not inhibit growth of either *Staph. aureus* 50084 or *Salm.* Typhimurium E224/87 at the highest concentration tested (higher concentrations were insoluble under our conditions), (ii) LC–MS analysis of the phenolic compounds in *S. papillosum* identified *p*-hydroxyacetophenone as the predominant phenolic (Table 2), at approx. threefold higher concentration than SA, (iii) MIC/MBC values for *p*-hydroxyacetophenone were lower than those of SA and assuming their MICs/MBCs represent preservative activity, we would predict *p*-hydroxyacetophenone to exert a greater antibacterial effect than SA. We note that the concentration of *p*-hydroxyl phenols in our batches of moss was between 40 and 170 times lower than that found by Williams *et al.* (1998). We believe that this derives from differences in methods of extraction and analysis employed. We chose LC–MS/MS instead of cupric oxide oxidation because mass spectrometry has greater discriminatory power than UV detection. Thus, it has the advantage that the ratio between different product ions provides additional identification and confirmation of the phenols, reducing the risk of false-positive and overestimated of results. Additionally, no derivatization is required. It is also known that *Sphagnum* species exhibit seasonal variation in SA content (Rudolph and Samland 1985).

The four minor phenolic components tested in the diffusion assay did not inhibit growth of *Staph. aureus* 50084, *B. cereus* NVH 0075/95 or *Salm.* Typhimurium E224/87. However, three of these phenols did show weak bacteriostatic activity against *S. putrefaciens* ATCC 8071. We have attempted to obtain MICs/MBCs of two of the phenols found in highest quantities (*p*-hydroxyacetophenone and *p*-hydroxybenzaldehyde) in *S. papillosum* but have not been able to obtain reproducible growth of the organism in TSB and therefore cannot define the extent to which these compounds may prevent spoilage organisms from multiplying.

In summary, we have quantified the amounts of phenolic compounds (including SA) present in leaves of *S. papillosum* and tested their antibacterial activities against food-borne pathogens. The microgram per ml concentrations of the three dominant phenolics were several orders of magnitude greater than that needed to exert antibacterial activity ($mg\ ml^{-1}$) at pH values appropriate for the undissociated forms. SA itself was without detectable effect. It is possible that phenolic compounds from *Sphagnum* moss are important in preservative action when present in environments of greater acidity than pH 5, but such conditions are difficult to test against food-borne neutrophilic bacteria in conventional antibacterial assays. Indeed, we have recently shown that the acid form of the polysaccharide sphagnan isolated from *Sphagnum* moss can inhibit growth of acid-sensitive bacterial species by lowering the pH (Stalheim *et al.* 2009). Thus, we suggest that the poor efficacy *in vitro* of moss-derived phenolics in intact leaves will not provide effective antibacterial action against food-borne pathogens.

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