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Colorimetric Assay to Determine *In Vitro* Antibacterial Activity Against Clinical Isolates: Enhanced Activity in Damaged Chinese Masson Pine Needles

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Abstract

A colorimetric assay for antibacterial susceptibility testing of clinical isolates (*Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Bacillus cereus*, and *Streptococcus pneumoniae*) is described based on the reduction of a novel tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS), in the presence of phenazine methosulfate (PMS) as an electron-coupling agent. The combination of 200 μg/mL MTS with 25 μmol/L PMS resulted in production of large amounts of formazan within 1 h of exposure. In this setting, fractions extracted from Chinese Masson pine (*Pinus massoniana* Lamb.) needles damaged by the pine caterpillar *Dendrolimus punctatus* Walker were found to have enhanced levels of antibacterial activity. These fractions, which were designated "Master", "Technique", and "Strength", were isolated and identified by reverse-phase C_{18} cartridge concentration, gel filtration, and affinity chromatography. Two fractions purified from healthy and undamaged needles were designated H1 and H2, respectively. For all test bacteria species. Technique produced the lowest minimal inhibitory concentrations (MICs), ranging from 2 to 32 μg/mL, and H2 produced the highest values, with four of the six MICs being higher than 128 μg/mL. We found that the R_{max} model fitted the data well in that the r^2 ranged between 0.87 and 0.96 (median, 0.92) and no statistically significant deviations from the model were found ($P = 0.23$). The median coefficient of variation of the log RC_{50} values and the slope m of the fitted model for all six strains among the replicates were 38 and 41%, respectively. In the course of the investigation, the physiological and functional factors involved in pest damage to plants were also explored. In summary, the MTS-PMS colorimetric assay has advantages over existing methods for the examination of antibacterial activity, and could be developed further such that it would be suitable for screening new antibiotic molecules.

Key words: antibacterial activity; clinical isolate; colorimetric assay; Masson pine needle; tetrazolium salt.

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There are two widely used methods for the determination of antibacterial activity: first, the two-layer radial diffusion method or inhibition zone assay (Lehrer et al. 1991) and, second, the colony-forming unit technique (Lassègues et al. 1989). These two methods sometimes fail to detect activity when the antibacterial substances have limited potency against the test microorganisms, or when experimental errors in measuring the inhibition zone or counting the bacterial colonies occur. Hence, there is a need for a new colorimetric method for testing antibacterial activity that is more sensitive and less prone to systematic errors.

The method described here relies on a novel tetrazolium salt,

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3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), acting in the presence of an electron coupling reagent, phenazine methosulfate (PMS). Tetrazolium salts are heterocyclic organic compounds that substitute for the natural final acceptor (oxygen) in the biological redox process and are reduced to formazan derivatives by receiving electrons enzymically from substances in the hydrogen transport system or nonenzymically from artificial electrontransporters (e.g. phenazine methosulfate) to enhance the reaction. The tetrazolium salt MTT has been used for antifungal susceptibility testing of various yeasts and filamentous fungi for years, and this method is recommended by the National Committee for Clinical Laboratory Standards (NCCLS; Clancy and Nguyen 1997; Meletiadis et al. 2000). The disadvantage of MTT, however, is that the process includes the solubilization of formazan derivatives by organic solvents such as dimethyl sulfoxide. Compared with MTT, MTS can penetrate more rapidly into intact cells, and is directly bioreduced into a colored formazan, which becomes water-soluble in a culture medium by receiving electrons from PMS. The absorbance of the formazan at 490 nm can be measured directly from 96-well assay plates, thereby avoiding the additional steps involved with solubilizing the formazan derivatives as required for MTT-based assays (Roehm et al. 1991; Meshulam et al. 1995). The conversion of MTS into aqueous soluble formazan is accomplished by the membranous dehydrogenase activity found in metabolically active cells. The quantity of formazan product, as measured by absorbance at 490 nm, is directly proportional to the number of viable cells in culture. Therefore, antibacterial susceptibility can be quantified on the basis of the difference in OD between the controls and tests in the presence of isolated antibacterial substances.

Although much research effort has been directed at using tetrazolium salts for antifungal susceptibility testing, which has resulted in clear-cut endpoints for various antifungal agents (Tellier et al. 1992; Hawser et al. 1998), there has been limited information published regarding antibacterial susceptibility testing with tetrazolium salts. In the case of MTS, the nature and the concentration of this agent used are critical in order to obtain a good correlation between formazan production and the number of viable bacteria, and less variable concentration-effect curves (Altman 1976; Tellier et al. 1992). Thus, we developed a new colorimetric assay to quantify the growth of six different bacterial species, including both Gram-negative and Gram-positive bacteria. The system is based on the tetrazolium salt MTS, and various factors that influence MTS conversion are standardized. We also investigated some active fractions that were isolated and purified by reverse-phase C_{18} cartridge concentration, gel filtration and affinity chromatography from phytogenic materials, Chinese Masson pine (*Pinus massoniana* Lamb.) needles after they had been damaged by the pine caterpillar *Dendrolimus punctatus* Walker, to confirm its suitability for the antibacterial susceptibility testing of clinical bacteria. In addition, the physiological and functional repercussions of pest damage of host plant were also explored.

Results

MTS concentration gradient assay

Conversion of MTS into aqueous soluble formazan by viable bacterial cells depended on the concentration of MTS and the exposure time, as well as the concentration of PMS (Figure 1). Of the three concentrations of MTS used, 200 μg/mL resulted in two times higher formazan production than 50 μg/mL, and approximately 1.5 times higher than 100 μg/mL. MTS was metabolized by all of the test bacteria species, even when no PMS was added, which could be determined by the highest background absorbances of 1.530 and 1.228 at 2 h of exposure to PMS after 24 h and 12 h of incubation, respectively. An irregularly shaped curve, characterized by a trapezoid shape, was observed for a concentration of 100 μg/mL with 1 h of exposure to PMS and 12 h of incubation.

Quantitative assay of bacterial viability

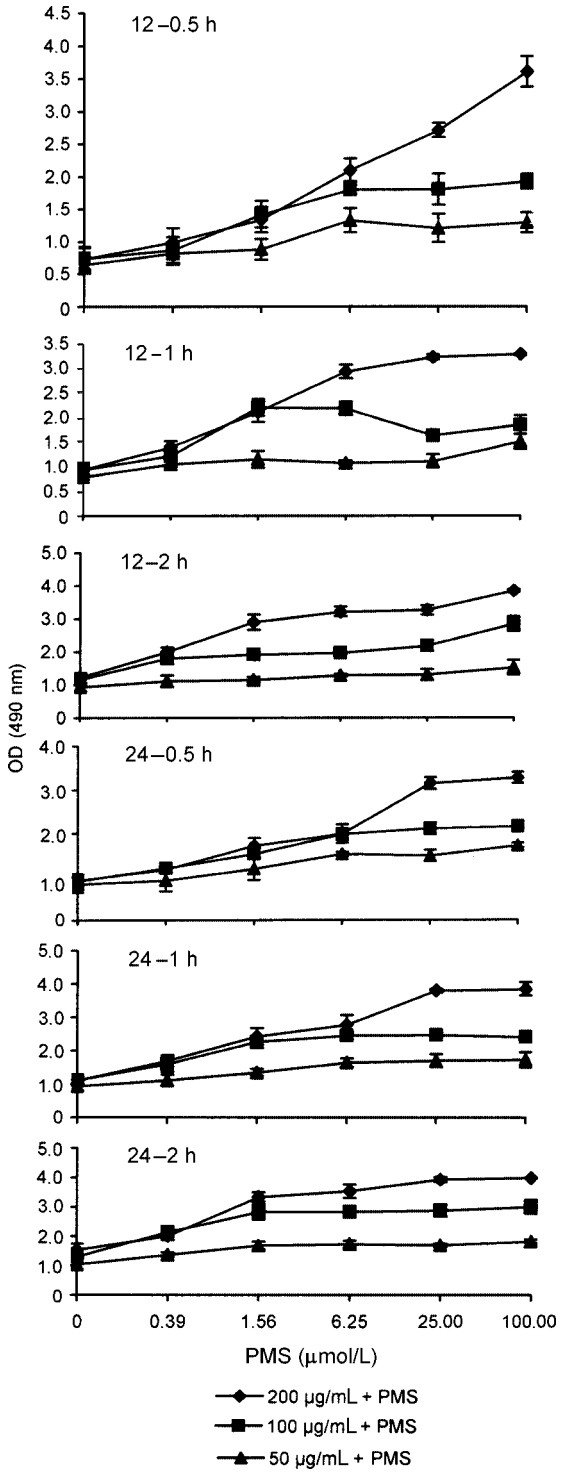
The correlation between MTS conversion and increasing inoculation of test bacteria was investigated using 200 μg/mL MTS with various concentrations of PMS after 24 h of incubation at 37 °C and 1 h of exposure. There was a linear relationship between the OD and log CFU (Figure 2; Table 1). The slopes and the coefficients obtained by regression analysis depended on the concentration of PMS used for each test bacterial strain. Generally, higher MTS conversion rates correlated with higher concentrations of PMS for all six strains (i.e. higher slopes were observed). However, at higher concentrations of PMS, for example 100 μmol/L, larger deviations from linearity were observed for all species (except *E. coli*) and shallower slopes were obtained for *P. aeruginosa* and *S. dysenteriae* (0.30 and 0.34, respectively; Table 1). Moreover, at a concentration of 100 μmol/L PMS, OD values occasionally exceeded the detection limit of the microplate reader, especially when inocula were exposed to MTS for 2 h after 24 h of incubation. Ideally, a concentration of PMS that ensures relatively high conversion rates and more balanced linear relationships between formazan production and viable bacteria number should be chosen as a standard parameter for further antibacterial activity assessments. Of the concentrations studied, 25 μmol/L PMS may be the best suited for this purpose (Table 1).

Antibacterial susceptibility assay

Five active fractions were obtained from Chinese Masson pine

needles by successive reverse-phase concentration, gel filtration, and affinity chromatography. Of these, three peaks were obtained from damaged needles by affinity elution with a stepwise gradient of 0.5, 1.0 and 1.5 mol/L NaCl, respectively. We named the elution volume fractions "Master", "Technique", and "Strength", respectively (i.e. peaks 1, 2, and 3 in Figure 3A). The other two fractions were purified from healthy or undamaged needles using a gradient of 0.5 and 1.0 mol/L NaCl, respectively, and were designated peak H1 and H2 (i.e. peaks 1 and 2 in Figure 3B). For all test bacteria species, Technique had the lowest minimal inhibitory concentration (MIC) values, ranging from 2 to 32 μg/mL, and H2 had the highest MIC values, of which four of six MIC values were higher than 128 μg/mL (Table 2). As shown in Figure 4, all concentration-effect curves based on MTS conversion had similar patterns and were characterized by two plateaus connected by a drop in relative OD. In the case of resistant strains, significant MTS conversion was observed at higher concentrations (e.g. *S. aureus* against Master, and *E. coli* against H2; Figure 4). The curves were analyzed using the R_{max} model with variable slope, and the best-fit values of the two variables RC_{50} and slope (m) were compared for each species, fraction, and incubation period. The model was found to fit the data well in that r^2 ranged between 0.87 and 0.96 (median, 0.92) and no statistically significant deviations from the model were found ($P = 0.23$). The median coefficient of variation of the log RC_{50} values and the slope m of the fitted model for all six strains were 38% and 41%, respectively.

The two-layer radial diffusion assay was chosen for comparison with the colorimetric method. For all active fractions and test species, higher MIC values were observed, and particularly with H1 no antibacterial activity was found in the concentration range of 0.125–128 μg/mL (Table 3). Activity titer curves obtained from the two-layer radial diffusion assay are shown in Figure 5. In general, a power function shape was observed for all curves, and the highest activity was obtained with Technique against *S. dysenteriae*, at approximately 46 mm^2. It is noteworthy that Technique was the only active fraction that was active for all test species.



Discussion

In this study we used a MTS-PMS colorimetric assay for distinguishing tiny differences between the number of viable bacteria number in tests and controls by linking bacteria dehydrogenase activity to the production of soluble colored formazan.

←

Figure 1. MTS reduction by test bacteria species.

Each of the test bacteria strains was incubated for 12 or 24 h and was then exposed for 0.5, 1.0, or 2.0 h to various concentrations of MTS (200 (diamonds), 100 (squares) and 50 (triangles) μg/mL), combined with PMS at various concentrations (0.39, 1.56, 6.25, 25.00, and 100.00 μmol/L). Each data point represents the average of all strains tested in triplicate.

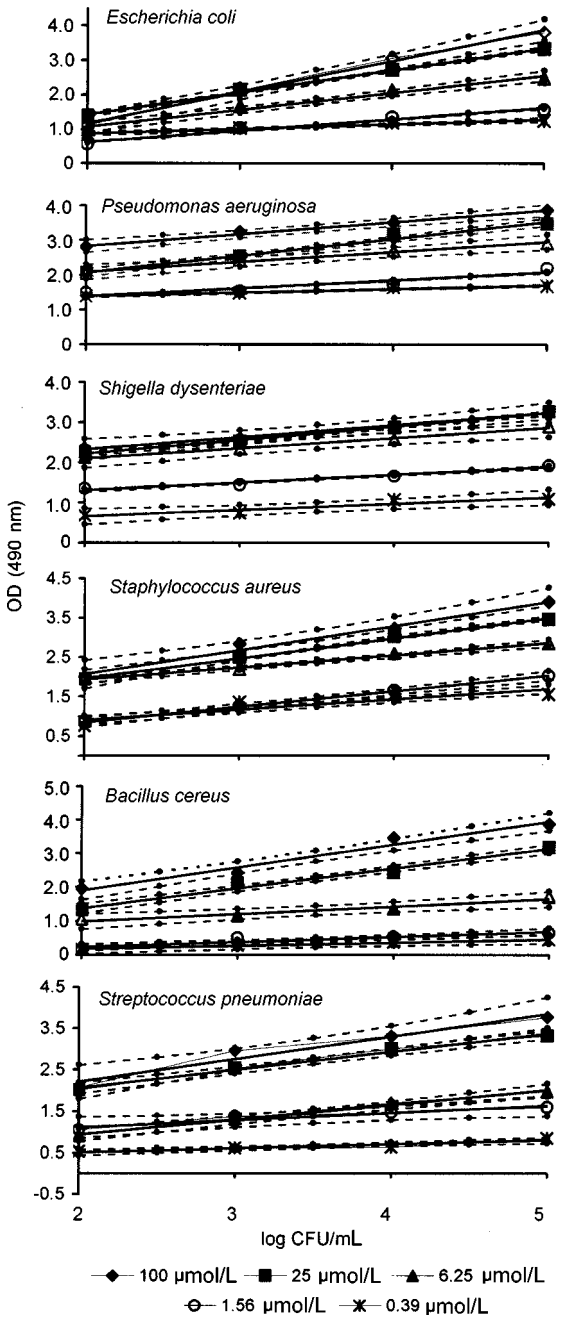


Figure 2. Relationship between the amount of MTS converted and increasing inocula of each test bacterial strain.

A concentration of 200 μg/mL MTS with various concentrations of PMS (100.00, 25.00, 6.25, 1.56, and 0.39 μmol/L) was used for triplicate tests of each strain. The solid lines were obtained by linear regression analysis, and the symbols stand for the means of the triplicates. Dotted lines represent the 95% confidence intervals of the regression lines.

The appeal of this method lies in its overcoming the shortcomings associated with the inhibition zone assay and the colony-forming unit technique. For inhibition zone assays, such factors as the thickness of the agar culture, the permeability and diffusion of antimicrobial substances, and measurement of the diameter of the inhibition zone may cause misleading results, even when precise measurements are carried out using vernier calipers. In the colony-forming unit technique, colony counting always involves errors. Hence, the MTS-PMS colorimetric assay was developed as a more robust and sensitive method for the determination of antimicrobial activity, by avoiding the intermediate steps involved in other methods. Using this methodology, enhanced antibacterial activity against clinical isolates was identified in extracts from damaged Chinese Masson pine needles, and the technical advantages of the MTS-PMS colorimetric assay with respect to sensitivity and spectrum were also confirmed relative to the ordinary two-layer radial diffusion assay (Tables 2,3).

The tetrazolium salt MTS has advantages over other alternatives. XTT, a tetrazolium compound similar to MTS, can also be bioreduced into an aqueous soluble formazan product (Paull et al. 1988). Unlike MTS, however, XTT has limited solubility and is not stable in solution. Procedures using XTT require daily preparation of fresh solutions using prewarmed (37 °C) or hot (60 °C) culture medium to produce a 1 mg/mL solution (Scudiero et al. 1988; Roehm et al. 1991). In contrast, the components of the MTS assay are supplied as filter-sterilized solutions in physiological buffered saline ready for use. Furthermore, because the MTS formazan product is soluble in tissue culture medium, the technique requires fewer steps than procedures that use tetrazolium compounds such as MTT or INT (Bernabei et al. 1989; CellTiter 1996). The formazan product of MTT reduction is a crystalline precipitate that requires an additional step in the procedure to dissolve the crystals before recording absorbance readings at 570 nm (Mosmann 1983).

The concentration of MTS is an important factor in the colorimetric assay that determines the rate of formazan production, especially at high concentrations (200 μg/mL). For the tetrazolium salt XTT, high concentrations may result in inhibition of formazan production, whereas very low concentrations can result in poor conversion (Tellier et al. 1992). For MTS, no inhibition was observed for the three concentrations tested in this study. The concentration of the electron-coupling agent PMS is another important factor that determines the linear relationship between formazan production using 200 μg/mL MTS and PMS with increasing inocula of each bacterial species. At a PMS concentration of 25 μmol/L, high coefficients and steep slopes for all test species were found, whereas PMS concentrations higher than 25 μmol/L generated a less linear CFU-OD relationship. Therefore, a concentration of 200 μg/mL MTS with addition of 25 μmol/L PMS was chosen for further studies.

Table 1. Relationship between amount of MTS reduction in the presence of different concentrations of PMS and increasing inocula of each test bacterial strain by linear regression analysis[a]

Strains	Relationship between PMS concentration and bacteria inoculum (log CFU/mL)									
	100 μmol/L		25 μmol/L		6.24 μmol/L		1.56 μmol/L		0.39 μmol/L	
	Slope	r^2	Slope	r^2	Slope	r^2	Slope	r^2	Slope	r^2
E. coli	0.89	0.996	0.64	0.974	0.49	0.923	0.33	0.862	0.13	0.705
P. aeruginosa	0.30	0.765	0.34	0.882	0.25	0.630	0.19	0.629	0.16	0.514
S. dysenteriae	0.34	0.545	0.49	0.985	0.29	0.916	0.23	0.737	0.11	0.401
S. aureus	0.61	0.836	0.54	0.957	0.31	0.542	0.39	0.661	0.26	0.538
B. cereus	0.68	0.802	0.59	0.976	0.22	0.863	0.15	0.841	0.09	0.336
S. pneumoniae	0.55	0.904	0.44	0.897	0.36	0.751	0.17	0.920	0.10	0.833

[a]Each replicate was analyzed as a separate point. OD and log CFU were used for calculations.

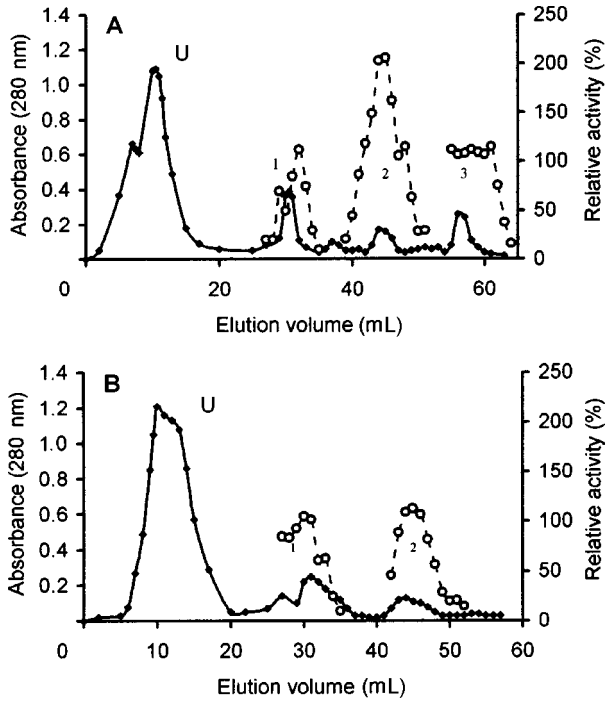


Figure 3. Final purifications of active fractions by affinity chromatography.

(A) Active fractions were purified from Chinese Masson pine needles that were damaged to a moderate extent (30%–50% needle loss) by *Dendrolimus punctatus* Walker pine caterpillars. Peaks 1, 2, and 3 (indicated by arrows), were obtained by elution with a stepwise NaCl gradient of 0.5, 1.0 and 1.5 mol/L in DPBS (pH 7.2), respectively. Peak U represents elution of unbound materials. The relative activity of each active peak was calculated (in percent) using the following formula: relative OD of synthetic cecropin A/relative OD of active fraction × 100%. Each data point represents the average of all strains tested in triplicate. **(B)** Active fractions were purified from healthy or undamaged pine needles. Peak U represents elution of unbound materials. Peaks 1 and 2 were obtained by elution with 0.5 and 1.0 mol/L NaCl in DPBS (pH 7.2), respectively. No activity was detected with a concentration of 1.5 mol/L NaCl in DPBS (pH 7.2).

The MTS-PMS assay was used to study the antibacterial activities of extracts from damaged and healthy pine needles. Of all isolated fractions, the active fraction Technique alone produced clear-cut endpoints, since MTS conversion was absent once the MIC was exceeded. For other active fractions, a general pattern of shallow curves was observed, either due to a partial inhibition of growth or possible interference of the active fraction in the metabolic process of the bacteria. Because resistant strains converted MTS even at high concentrations of the active fraction, which is similar to the situation with XTT (Meletiadis et al. 2001), discrimination between susceptible and resistant strains may be facilitated by using the MTS assay.

The most valuable information obtained from analyzing the enhanced antibacterial activities of damaged Chinese Masson pine needles is that over a long period of adaptive co-evolution, plants can develop delicate and complicated tactics to modulate the damage caused by insects. When plants are threatened by insects, they may alter their chemical content, and some secondary metabolites, such as phenols, tannins, and terpenoids, may be induced to defend the plant against the pests (Ge 1992). Previous work revealed that defoliation by insects not only lowers the leaf quality of plants, but also stimulates their innate immunity to herbivore attack (Cook 1961; Haukioja and Niemelä 1979; Shultz and Baldwin 1982; Raupp and Denno 1984; Su et al. 1993). This chemical resistance can be classified according to whether it induces a short-term or a long-term reaction, of which the former appears to influence the population dynamics of the parental insects immediately, whereas the latter may have an impact on later generations (Ge et al. 1997; Wang et al. 2001). An alternative explanation for the unsuitability of damaged plant leaves as food for insects are changes in leaf nutrition, moisture content, structure or allelochemistry that may interfere with herbivore growth and development (Raupp and Denno 1984). Not only can these changes in chemical production reduce food quality, retard pest growth, and decrease adult fecundity, but they can also make the pests more susceptible to disease, parasites, and

Table 2. Results of antibacterial susceptibility assay obtained by nonlinear regression analysis[a]

Active fraction and incubation time	Strains	Colorimetric assay		
		MIC[b] (μg/mL)	RC_{50} ± 95%CI (μg/mL)	Slope (*m*) ± 95%CI
M, 12 h	*E. coli*	16–32	6.42 ± 1.28	–1.36 ± 0.37
	P. aeruginosa	32–64	4.73 ± 0.59	–1.28 ± 0.65
	S. dysenteriae	8–16	0.84 ± 0.16	–1.72 ± 0.44
	S. aureus	>128	>128	–0.26 ± 0.12
	B. cereus	8–16	0.69 ± 0.24	–1.05 ± 0.46
	S. pneumoniae	32–64	3.71 ± 0.92	–0.83 ± 0.26
M, 24 h	*E. coli*	32–64	4.68 ± 0.76	–1.42 ± 0.43
	P. aeruginosa	32–64	3.31 ± 1.64	–1.54 ± 0.61
	S. dysenteriae	8–16	2.65 ± 0.80	–1.63 ± 0.47
	S. aureus	>128	>128	–0.39 ± 0.21
	B. cereus	16–32	3.89 ± 0.97	–1.30 ± 0.45
	S. pneumoniae	32–64	6.85 ± 2.92	–1.23 ± 0.38
T, 12 h	*E. coli*	2–4	1.65 ± 0.18	–3.07 ± 1.21
	P. aeruginosa	2–4	1.48 ± 1.21	–2.39 ± 0.85
	S. dysenteriae	16–32	22.47 ± 3.88	–8.21 ± 2.83
	S. aureus	4–8	3.91 ± 1.62	–3.78 ± 0.69
	B. cereus	8–16	7.93 ± 2.71	–6.56 ± 4.45
	S. pneumoniae	2–4	1.67 ± 0.25	–2.17 ± 0.90
T, 24 h	*E. coli*	2–4	1.62 ± 0.22	–5.26 ± 1.03
	P. aeruginosa	8–16	6.23 ± 2.86	–3.38 ± 1.43
	S. dysenteriae	16–32	23.05 ± 3.65	–8.59 ± 2.72
	S. aureus	8–16	10.41 ± 3.47	–3.25 ± 2.21
	B. cereus	8–16	8.01 ± 1.07	–5.84 ± 2.60
	S. pneumoniae	8–16	7.44 ± 2.98	–2.81 ± 0.33
S, 12 h	*E. coli*	4–8	2.25 ± 0.98	–1.75 ± 0.40
	P. aeruginosa	8–16	5.11 ± 1.29	–7.32 ± 2.16
	S. dysenteriae	8–16	5.94 ± 1.34	–1.36 ± 0.54
	S. aureus	8–16	5.07 ± 1.72	–1.09 ± 0.83
	B. cereus	64–128	28.33 ± 8.21	–0.99 ± 0.65
	S. pneumoniae	2–4	1.80 ± 0.67	–3.53 ± 2.41
S, 24 h	*E. coli*	8–16	3.70 ± 0.64	–1.84 ± 0.17
	P. aeruginosa	8–16	10.02 ± 1.43	–6.97 ± 3.01
	S. dysenteriae	16–32	13.66 ± 8.54	–1.14 ± 0.22
	S. aureus	16–32	15.35 ± 7.91	–2.33 ± 1.18
	B. cereus	>128	>128	–0.06 ± 0.03
	S. pneumoniae	8–16	5.76 ± 1.97	–2.68 ± 0.85
H1, 12 h	*E. coli*	16–32	22.13 ± 3.50	–1.00 ± 0.54
	P. aeruginosa	64–128	36.45 ± 10.57	–2.29 ± 0.82
	S. dysenteriae	>128	3.72 ± 2.35	–0.34 ± 0.07
	S. aureus	>128	>128	–0.10 ± 0.03
	B. cereus	16–32	9.64 ± 1.38	–1.88 ± 0.41
	S. pneumoniae	16–32	1.51 ± 0.59	–1.31 ± 0.35

Table 2. (continued)

Active fraction and incubation time	Strains	Colorimetric assay		
		MIC[b] (μg/mL)	RC_{50} ± 95%CI (μg/mL)	Slope (*m*) ± 95%CI
H1, 24 h	*E. coli*	16–32	7.46 ± 2.21	–3.18 ± 1.93
	P. aeruginosa	>128	>128	–0.23 ± 0.14
	S. dysenteriae	>128	>128	–0.15 ± 0.02
	S. aureus	>128	>128	–0.07 ± 0.02
	B. cereus	16–32	12.0 ± 6.15	–2.17 ± 0.75
	S. pneumoniae	32–64	11.3 ± 3.61	–1.21 ± 0.40
H2, 12 h	*E. coli*	>128	>128	–0.15 ± 0.04
	P. aeruginosa	>128	>128	–0.01 ± 0.00
	S. dysenteriae	8–16	2.93 ± 1.27	–2.63 ± 1.10
	S. aureus	64–128	81.2 ± 43.9	–1.48 ± 0.45
	B. cereus	16–32	13.7 ± 3.95	–1.40 ± 0.25
	S. pneumoniae	>128	>128	–0.06 ± 0.01
H2, 24 h	*E. coli*	>128	>128	–0.04 ± 0.01
	P. aeruginosa	>128	>128	–0.01 ± 0.00
	S. dysenteriae	8–16	6.44 ± 2.78	–2.46 ± 0.87
	S. aureus	>128	>128	–0.13 ± 0.02
	B. cereus	32–64	51.8 ± 16.4	–1.52 ± 0.74
	S. pneumoniae	>128	>128	–0.02 ± 0.01

[a]Data were obtained by nonlinear regression analysis using a four parameter logistic model with variable slopes for the concentration effet curves obtained by colorimetric (MTS) method after 12 and 24 h of incubation with serial two-fold dilutions of active fractions of M (Master), T (Technique), and S (Strength), and active fractions of H1 and H2, which were isolated from healthy pine needles and used as controls. 95% CI stands for 95% confidence intervals.

[b]Minimal inhibitory concentrations (MICs) were determined with serial dilutions of each active fraction in a 96-well microtiter plate, and are expressed as an interval, a–b, where a represents the highest concentration tested at which bacteria still grow and b is the lowest concentration that inhibits the cell growth.

predators (Price et al. 1980). Although our results do not provide a link between enhanced antibacterial activity in damaged pine needles and their chemical resistance to pests, it is tempting to surmise that the herbivory-induced resistance of damaged host plants, followed by herbivore population decline and relaxation of plant responses could coincide with the development of antibacterial activity by coexpression of corresponding genes, or linked characters, in host plants.

In summary, we have shown that the MTS-PMS system can be used to evaluate the antibacterial activity of purified active fractions from natural resources. Compared with alternative methods, the MTS-PMS assay appears to be faster and more sensitive and convenient. Although pine needles have been used as anti-inflammatory agents in traditional Chinese medicine, and a couple of studies have found antibacterial activity in extracts from some pine species (Xiao and Ren 1994; Li et al. 1999), in the present study enhanced antibacterial activity against clinical isolates was found in three fractions from damaged Chinese Masson pine needles by using the MTS-PMS assay. To our knowledge, this is one of the first studies with the aim of elucidating the relationships between damage-induced antibacterial molecules and chemical defenses against herbivorous insects in host plants, and represents a new attempt to carry out high-throughput screening of novel antibacterial agents of plant origin. Further work will focus on the development of a tetrazolium salt that might not require the addition of an electron-coupling agent, because the need for an electron-coupling agent complicates the assay and may increase variability (Scudiero et al. 1988).

Materials and methods

Test bacteria species

Three strains of Gram-negative bacteria, including one each of *Escherichia coli* (E-109), *Pseudomonas aeruginosa* (AH-1509), and *Shigella dysenteriae* (AH-502) (obtained from clinical isolates from the PLA 306 Hospital), and three strains of Gram-positive bacteria, including one each of *Staphylococcus aureus*

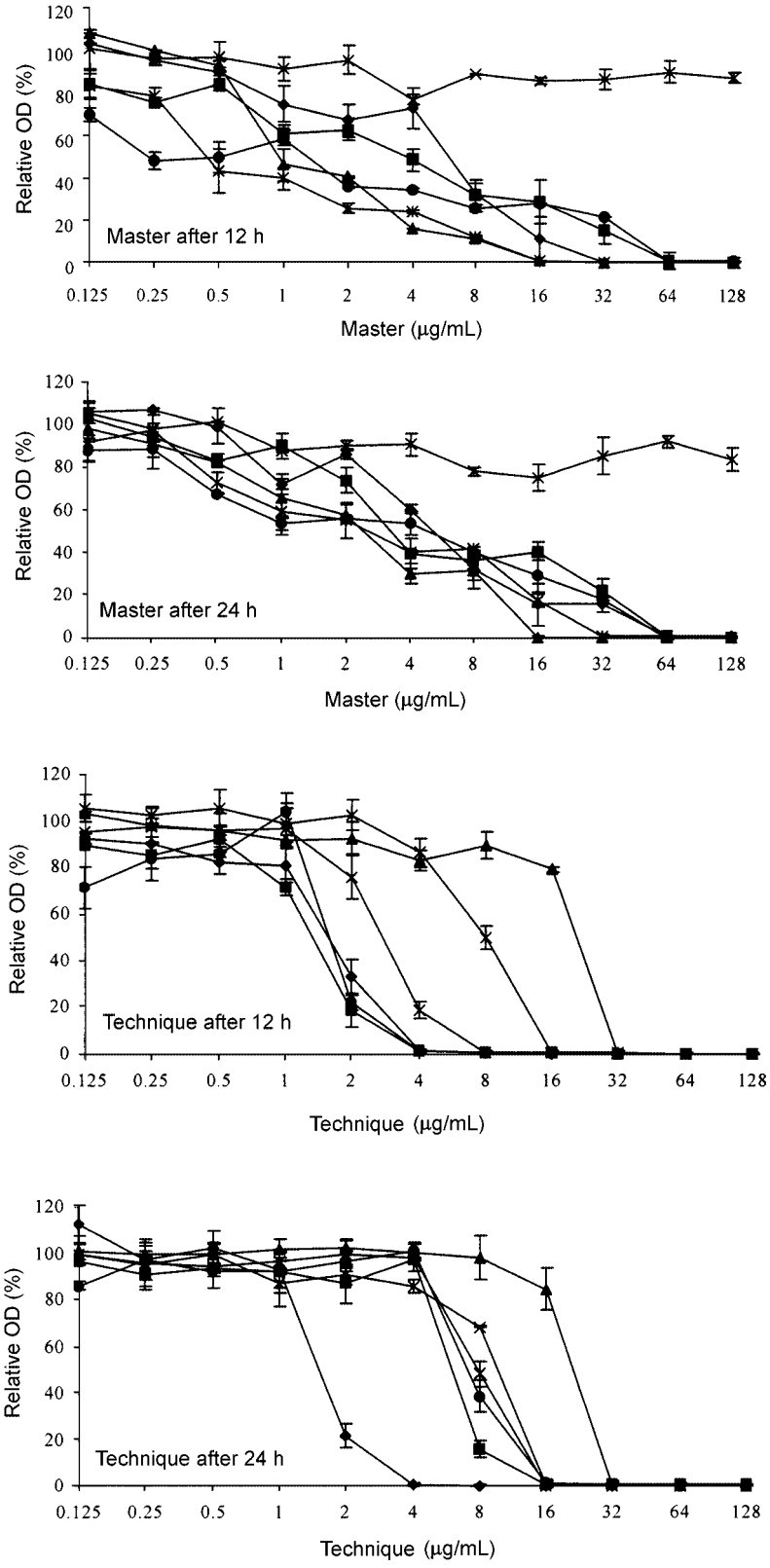


Figure 4.

(26110), *Bacillus cereus* (B-256), and *Streptococcus pneumoniae* (SP-3124), were used in the present study. *Escherichia coli* ((Migula) Castellani et Chalmers) (CGMCC 1.154 3) and *Staphylococcus aureus* (Rosenbach)(CMCC (B) 26001) were used for quality control. All bacteria except *S. pneumoniae* were grown in a sterile liquid nutrient broth (LNB) made from 10 g of pancreatic peptone, 3 g of meat extract, and 5 g/L NaCl of distilled water adjusted to pH 7.0 and used during the exponential growth phase. Penessay agar plate or broth was used for the subculture of *S. pneumoniae*. The bacterial concentrations were adjusted to an inoculum size of 10^6 CFU/mL in fresh sterile saline containing 0.05% Tween 20. The inoculum size was confirmed by serial dilutions on corresponding plates.

Medium, MTS and PMS preparation

RPMI-1640 medium (with *L*-glutamine and without bicarbonate) was prepared as previously described (Pan et al. 2003). Briefly, RPMI-1640 medium was buffered to pH 7.0 with 0.165 mol/L 3-N-morpho-linopropanesulfonic acid (MOPS) and used throughout.

CellTiter 96 AQ_{ueous} MTS powder (Promega Corporation, Madison, WI, USA) and PMS (Sigma-Aldrich Chemie) were dissolved in Dulbecco's phosphate-buffered saline (DPBS) at final concentrations of 1.2 mg/mL and 600 μmol/L, respectively. The solutions were filtered through a filter with 0.22-μm pores. Further dilutions of MTS or PMS, if needed, were made using DPBS.

Harvesting of active fractions

Freshly collected needles from Chinese Masson pine (*Pinus massoniana* Lamb.), either pine trees damaged to a moderate extent (30–50% needle loss) by *Dendrolimus punctatus* Walker pine caterpillar or healthy trees used as controls, were immersed and cleaned with cool water, and frozen at –196 °C in liquid nitrogen for 1 h. The needles were cut into small pieces and ground to fine powder with liquid nitrogen. Approximately 20 g pine powder was weighed and mixed with 100 mL of acidic buffer solution containing 1% (v/v) trifluoroacetic acid, 1 mol/L HCl, 5% (v/v)

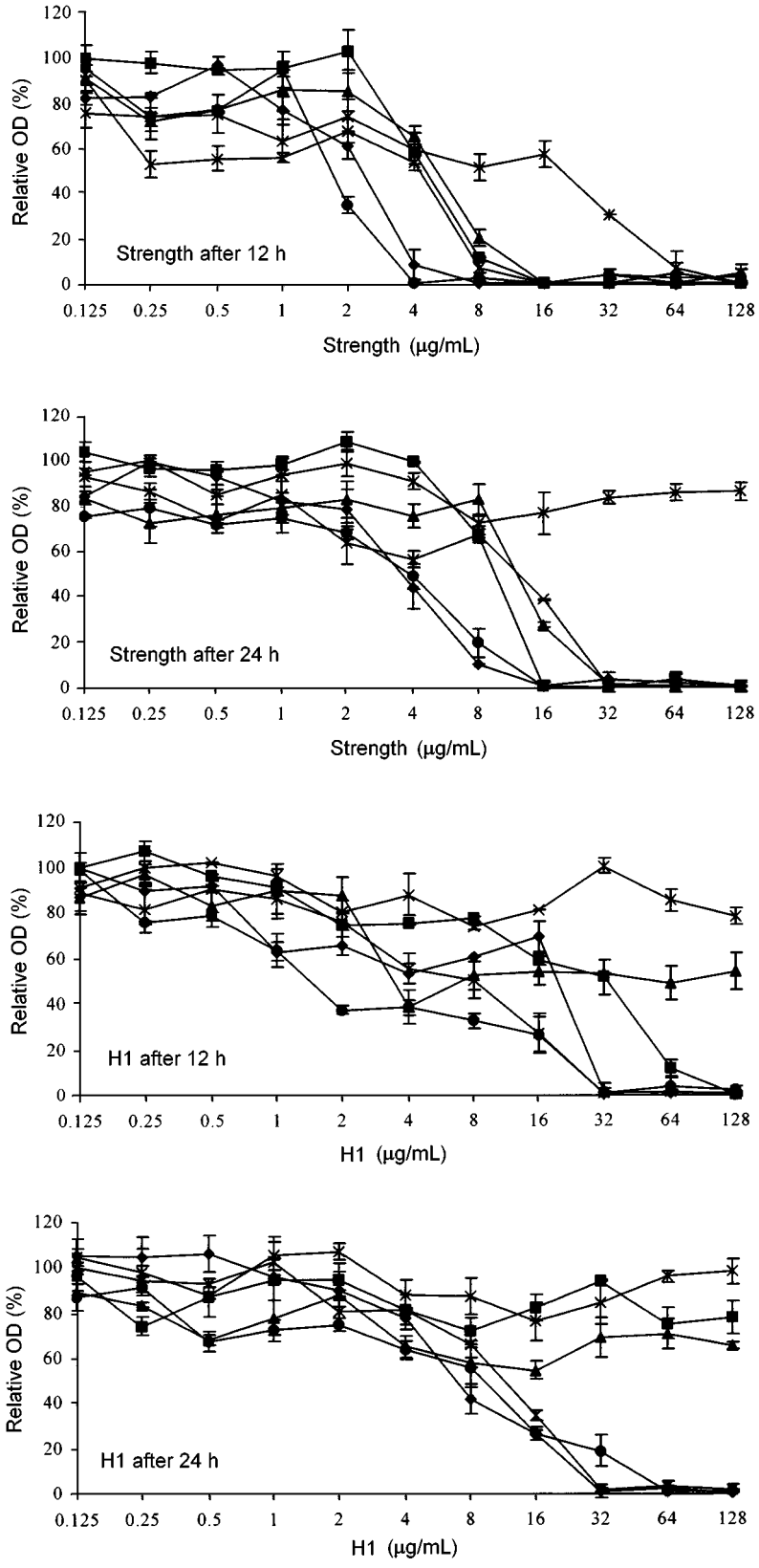


Figure 4.

formic acid, 1% (w/v) NaCl and pepstatin A at a concentration of 1 μg/mL. After filtration, the filtrate was redissolved twice in the same acidic buffer solution and the whole filtrate was gathered and centrifuged at 4 °C, 50 000 *g*, for 30 min, and the supernatant was collected. The active substances in the supernatant were then placed in a Sep-Pak C_{18} cartridge (Waters Associates, Milford, USA) for reverse-phase concentration and the eluates were loaded onto a Sephadex G-100 column (1.6 cm × 90 cm) with 0.02 mol/L Tris-HCl (pH 8.0) at a flow rate of 7 mL/min for elution. The active fractions were reloaded onto a 1.6 cm × 2.5 cm Hitrap Heparin HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) for affinity separation with a stepwise NaCl gradient of 0.5, 1 and 1.5 mol/L in DPBS (pH 7.2). The active fractions were pooled, desalted and concentrated by ultrafiltration, and lyophilized.

Concentration gradient assay for MTS

Two-hundred microliters of each bacterial suspension of test species (diluted to 10^4 CFU/mL with the RPMI-1640 medium) was inoculated into 96-well flat-bottomed microtiter plates (F96 microtiter plates; Nunc, Denmark). After 12 or 24 h of incubation at 37 °C, 50-μL aliquots of various concentrations of MTS and 50-μL aliquots of various concentrations of PMS were added to the wells in order to obtain final concentrations of 200, 100, and 50 μg of MTS/mL and 100, 25, 6.25, 1.56, and 0.39 μmol/L PMS. The microtiter plates were incubated further for 2 h at 37 °C and OD at 490 nm was measured by using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA) at time intervals of 0.5, 1, and 2 h. The MTS assay was performed for each species in triplicate and ODs for different time intervals or concentration gradients were analyzed.

Quantitative assay of bacterial viability

To standardize the relationship between the number of viable bacteria and the amount of MTS reduction, various inocula of test bacteria (10^2 to 10^6 CFU/mL) were incubated with MTS and various concentrations of PMS. The 96-well flat-bottomed microtiter plates were inoculated with 200 μL of each bacterial dilution

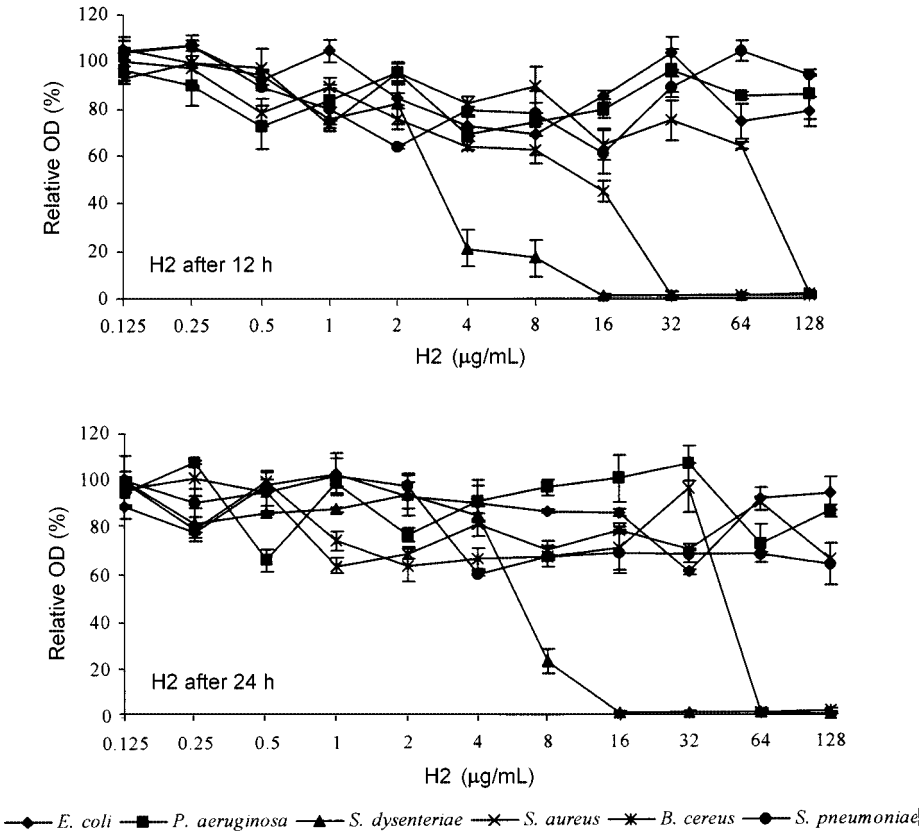


Figure 4. Concentration-effect curves for active fractions of Master, Technique, Strength, and H1 and H2 (controls).

Each bacterial strain was incubated for 12 or 24 h and exposed to 200 μg/mL MTS plus 25 μmol/L PMS for 1 h, and then the OD value at 490 nm was measured. Relative ODs for the concentration of each active fraction represent the amount of formazan produced in comparison with the growth control. Means (± *SE*) of triplicate experiments are shown for each strain.

Table 3. Antibacterial activities of active fractions according to the two-layer radial diffusion assay

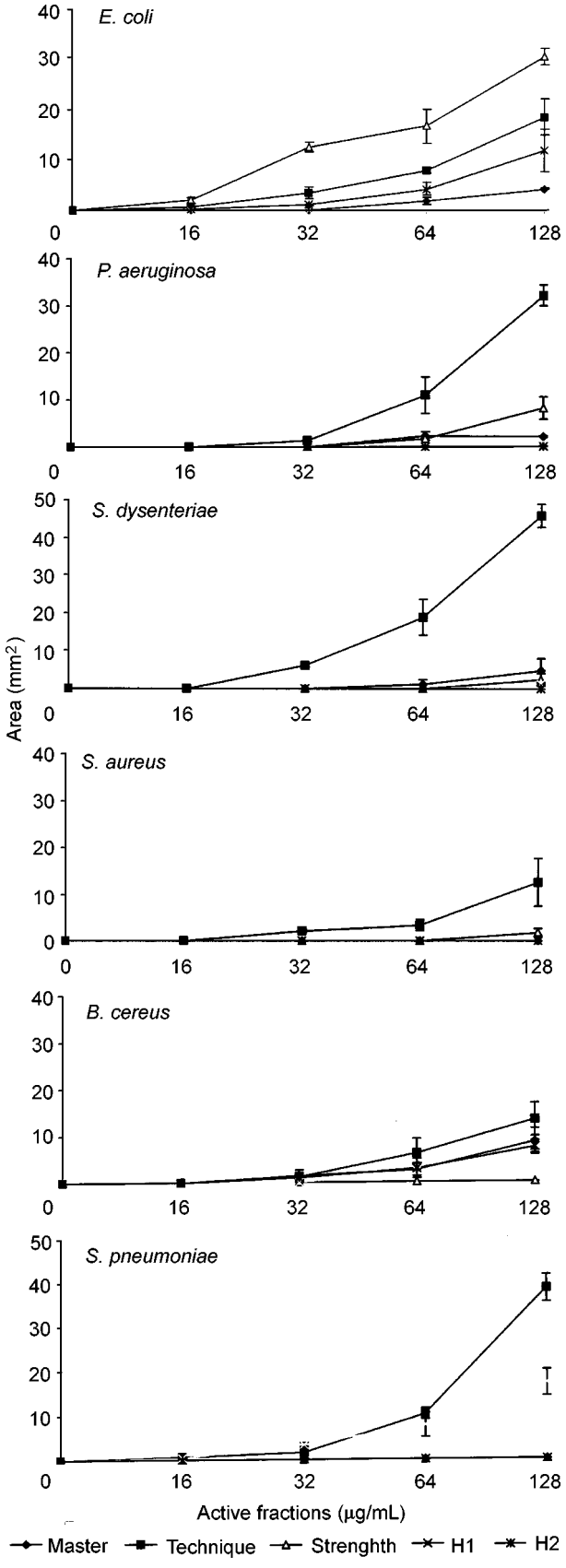
Strains	MIC[a] (μg/mL)				
	Master	Technique	Strength	H1	H2
E. coli	32–64	8–16	4–8	>128	8–16
P. aeruginosa	>128	2–4	32–64	>128	>128
S. dysenteriae	32–64	>128	8–16	>128	>128
S. aureus	>128	8–16	>128	>128	>128
B. cereus	>128	16–32	>128	>128	>128
S. pneumoniae	16–32	64–128	>128	>128	>128

[a]MICs were determined by serial dilutions of active fractions that inhibit the growth of bacteria around 2-mm diameter wells, and are expressed as an interval, *a*–*b*, where *a* represents the highest concentration at which bacterial colonies still form and *b* is the lowest concentration that prevents the formation of visible bacteria colonies.

series (from 10^2 to 10^6 CFU/mL). After 24 h of incubation at 37 °C, 50-μL aliquots of MTS and 50-μL aliquots of various concentrations of PMS were added to each well as to obtain final concentrations of 200 μg/mL for MTS and 100, 25, 6.25, 1.56, and 0.39 μmol/L for PMS. The microtiter plates were then incubated for another 1 h, after which OD at 490 nm was measured. This experiment was carried out in triplicate for each bacterial strain, and data analysis was performed by linear regression analysis. Regression lines with 95% confidence intervals were plotted for different levels of PMS and for each bacterial strain.

Antibacterial susceptibility assay

Lyophilized active fractions purified from pine needles were reconstituted in DPBS (pH 7.2). The protein levels were determined for each fraction by using the method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard. Protein concentrations based on determinations were diluted twofold in RPMI-1640 medium to obtain a final concentration range from 0.125 to 128 μg/mL. Each well in 96-well microtiter plates was filled with 100 μL medium, 50 μL of each strain suspension (1–2×10^4 CFU/mL), and 50 μL of each active fraction or synthetic cecropin A. The synthetic antibacterial peptide cecropin A, which also ranged in concentration from 0.125 to 128 μg/mL, was used as a quality control in this test. An active fraction-free well containing 50 μL of the same volume of DPBS diluted in the medium served as the growth control. Background OD was measured using bacteria-free wells processed in the same way as the bacteria-containing wells. The plates were incubated for 12 or 24 h at 37 °C with gentle stirring, and then 25 μL of MTS and 25 μL of PMS were added to each well as to obtain final concentrations of 200 μg/mL and 25 μmol/L, respectively. After a further 1 h incubation, the OD of each well at 490 nm was measured for colorimetric quantification of soluble formazan production. The relative OD for each well, based on measurements at 490 nm, was calculated (in percent) using the following equation: (OD of antibacterial dilution containing well-background OD) / (OD of growth



control well-background OD of growth control well) × 100%. The relative activity of each active fraction was calculated (in percent) using the following formula: relative ODs of cecropin A / relative ODs of active fraction × 100%. The tests were performed in triplicate in three independent experiments for each bacterial strain. The results from each experiment were analyzed by nonlinear regression analysis as described by Meletiadis et al. (2001). Briefly, a four-parameter logistic model (sigmoid curve with variable slope) was used with the following equation: $R = R_{max} \times (AF/RC_{50})^m/(1+(AF/RC_{50})^m)$, where R is the relative OD (dependent variable), R_{max} is the maximum relative OD, AF is the concentration of active fraction (independent variable), RC_{50} is the concentration of active fraction producing 50% of the R_{max}, and m is the slope that describes the steepness of the curve (Greco et al. 1995).

Determination of minimal inhibitory concentrations of active fractions

The MICs, described previously by Moore et al. (1991), were improved and determined with serial dilutions of the purified active fractions in a 96-well microtiter plate, and estimated from the absorbance at 490 nm after incubation with the MTS-PMS mixture. The MIC values were expressed according to Casteels et al. (1993) as an interval, *a–b*, where *a* represents the highest concentration tested at which the bacteria were still growing and *b* is the lowest concentration that inhibits cell growth. In fact, it indicates that no antibacterial activity is available while the relative OD values are equal to or more than 100%.

Two-layer radial diffusion assay

In order to compare the antibacterial effects detected by the two-layer radial diffusion and colorimetric methods, the two-layer radial diffusion test according to the method of Lehrer et al. (1991) was also performed. Briefly, this entailed preparing twofold serial dilutions of each active fraction (0.125 to 128 μg/mL) for each 10-cm^2 sterile Petri dish. Wells 2 mm in diameter were punched in the agarose and 10-μL volumes of active fractions were pipetted into each. The diameters of clear zones in the agar underlays were measured under a binocular microscope and activity was expressed as clear zone area (in mm^2)

←

Figure 5. Titer of activity of five isolated fractions from *Pinus massoniana* Lamb. needles against bacterial strains.

Each purified protein with twofold serial dilutions (0.125 to 128 μg/mL) in distilled water was assayed against an individual test strain by using a two-layer radial diffusion assay. Data are means ± *SE* of measurements carried out in triplicate for three independent experiments.

minus the area of the well. This assay was carried out for each species in triplicate in three independent experiments, and activity titer curves were plotted.

Statistical analysis

All tests in the study except where specified otherwise were performed for each species in triplicate and in three independent experiments, and ODs for different time intervals or concentration gradients were analyzed. The slopes and the r^2 of each regression line were recorded for the estimation of steepness of the line and goodness of fit, respectively. An r^2 value of 1 indicates perfect correlation. The nonlinearity of the curves was checked by using a runs test following linear regression. Analysis was carried out using SPSS 12.0 for Windows (Chicago, IL, USA). Deviation from the model was tested by the runs test, and goodness of fit was checked by using the r^2 values. The best-fit values of RC_{50} and slope (m) resulting from the nonlinear regression analysis were used to compare the concentration-effect curves. A significance level of 0.05 was taken as being statistically significant.

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