Reconfirmation of antimicrobial activity in the coelomic fluid of the earthworm *Eisenia fetida andrei* by colorimetric assay

WEIDONG PAN[†], XIANGHUI LIU, FENG GE* and TAO ZHENG[§]

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, People's Republic of China

[§]*Bioelectromagnetic Laboratory, Institute of Electrical Engineering, Chinese Academy of Sciences, Beijing, People's Republic of China*

[†]*Present address: PO Box 27, Institute of Zoology, Beisihuan West Road 25, Beijing 100080, People's Republic of China*

**Corresponding author (Fax, 86-10-62565689; Email, gef@panda.ioz.ac.cn)*

A novel tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) was used in the assessment of antimicrobial activity in earthworm in the presence of phenazine methosulphate (PMS) as an electron coupling reagent. This activity was purified from the coelomic fluid of the earthworm (ECF), *Eisenia fetida andrei* (Oligochaeta, Lumbricidae, annelids) using a series of column chromatography techniques and was tested against three Gram-negative strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and three Gram-positive strains of *Staphylococcus aureus*, *Bacillus megaterium*, *Arthrobacter* sp., respectively. Only the pigment-free eluate of coelomic fluid of the earthworm (ECFPE) showed activity against *B. megaterium* amongst three isolated active fractions. The anion (DEAE-52) exchange effluent of the ECFPE was reported to have the strongest activity against *P. aeruginosa* amongst the three active fractions. The 20% acetonitrile eluate (AE) by Sep-Pak C_{18} cartridge was also tested and showed fair resistance against *E. coli*, *P. aeruginosa* and *Arthrobacter* sp., respectively.

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1. Introduction

There have been many investigations on the presence of antimicrobial substances in annelids since the first bacteriolytic molecules were identified as lysozyme-like molecules, active only against Gram-positive bacteria (Jollés and Zuili 1960; Schubert and Messner 1971; Lassègues 1986). The coelomic fluid of the earthworm, *Eisenia fetida andrei* (Savigny) (Oligochaeta, Lumbricidae) was demonstrated to possess an antimicrobial activity directed against earthworm pathogenic bacteria – namely; Gram-negative *Aeromonas hydrophila* [(Chester) Stanier] and Gram-positive *Bacillus megaterium* (de Bary) by Valembois *et al* (1982). Later, Milochau *et al* (1997) purified two proteins, named fetidins, from dialysed coelomic fluid of the earthworm and confirmed that the antimicrobial activity existing in the coelomic fluid was essentially due to fetidins.

Nevertheless, the widely used methods for the determination of antimicrobial activity were confined to inhibition-zone assays with a modification of the two-layer radial diffusion method (Lehrer *et al* 1991) and the colony-forming unit technique (Lassègues *et al* 1989). These two methods, although having merits, can bring errors into

Keywords. Antimicrobial activity; column chromatography; earthworm; *Eisenia fetida andrei*; Tetrazolium salt

Abbreviations used: AE, Acetonitrile eluate; DPBS, Dulbecco's phosphate buffered saline; ECF, coelomic fluid of the earthworms; ECFPE, ECF pigment-free eluate; MICs, minimum inhibitory concentrations; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; PMS, phenazine methosulphate.

activity assessments. When the antimicrobial substances have limited potency against test microorganisms, the inhibition-zone assays or the colony-forming unit technique often can not make a difference between the tests and controls. Such experimental manipulations as the inhibition-zone measuring and the bacteria colony numbering may sometimes lead to big experimental errors as well. Hence, arose a need for a new colorimetric method which highlights more sensitivity and less systematic errors.

The colorimetric method depends on a novel tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) acting in the presence of an electron coupling reagent phenazine methosulphate (PMS). MTS can penetrate into intact cells and are bioreduced by receiving electrons from PMS into a coloured formazan which is soluble in a culture medium. The absorbance of the formazan at 490 nm can be measured directly from 96 well assay plates without any additional solubilization process as with MTT. The conversion of MTS into aqueous-soluble formazan is accomplished by membranous dehydrogenase activity found in metabolically active cells. The quantity of formazan product – as measured by the amount of 490 nm absorbance – is directly proportional to the number of viable cells in culture. Therefore, the amount of antimicrobial susceptibility can be calculated from the OD difference between the controls and tests in the presence of isolated antimicrobial substances.

To confirm the feasibility of this method, we investigated some active fractions isolated from the coelomic fluid of the earthworm *E. f. andrei* by reverse-phase C_{18} cartridge, gel filtration and anion exchange chromatragraphy, respectively. Although a couple of antimicrobial activities as lysozyme and fetidins (Lassalle *et al* 1998; Milochau *et al* 1997) had been located in the coelomic fluid of the earthworm *E. f. andrei*, we identified novel antimicrobial activities against six test bacteria strains by MTS-PMS assay and made comparisons of their activity amount as well.

2. Materials and methods

2.1 *Harvesting of coelomic fluid of the earthworm*

Earthworms, *E. f. andrei* (Savigny) (Oligochaeta, Lumbricidae) were purchased from Northwest Suburb Earthworm Company, Beijing, Chaina. The earthworms were immersed in clean, cool water for 48 h to eliminate gastrointestinal metabolites and contaminants. They were then rinsed and rapidly dried on a filter paper, and were subsequently excited with 5 V electrical stimulations to produce coelomic fluid through their epidermal dorsal pores (Roch 1979). After centrifugation for 10 min at 4°C, 12,000 *g* in a Himac CP70G (Hitachi, Tokyo, Japan), the cell-free supernatant of coelomic fluid was collected and sterilized with 0·20 μm Minisart® filter (Sartorius AG, Goettingen, Germany) for immediate use or stored at – 20°C until needed.

2.2 *Test bacteria species*

Three strains of Gram-negative bacteria: namely; *Escherichia coli* [(Migula) Castellani et Chalmers] (CGMCC 1·1543, Ap^rTc^sT4G30), *Pseudomonas aeruginosa* [(Schroeter) Migula] (CGMCC 1·50), *Aeromonas hydrophila* [(Chester) Stanier] (CGMCC 1·1814) and three strains of Gram-positive bacteria: namely; *Staphylococcus aureus* (Rosenbach) [CMCC (B) 26001], *Bacillus megaterium* (de Bary) (CGMCC 1·151), *Arthrobacter* sp. (CGMCC 1·8) were selected. All bacteria were grown in sterile liquid nutrient broth (LNB) made from 10 *g* of pancreatic peptone, 3 *g* of meat extract, and 5 *g* of NaCl/l of distilled water adjusted at pH 7·0 and used at the exponential growth phase. The bacterial concentrations were finally adjusted to an inoculum size of 10^6 CFU/ml in fresh sterile saline. The inoculum size was confirmed by plate serial dilutions on nutrient broth plates.

2.3 *Medium and MTS/PMS preparation*

The RPMI 1640 medium was prepared according to Meletiadis *et al* (2001). A RPMI 1640 medium buffered to pH 7·0 with 0·165 M 3-N-morpho-linopropanesulphonic acid (MOPS) was used throughout.

The combined MTS/PMS solution was obtained with a CellTiter 96®AQ_{ueous} MTS Reagent Powder (Promega Corporation, Madison, WI, USA) and PMS (Sigma-Aldrich Chemie GmbH) dissolved in Dulbecco's phosphate buffered saline (DPBS) at a final concentration of 2·5 mg/ml for MTS and 312·5 μM for PMS. Further, dilutions of MTS/PMS, if needed, were made in RPMI 1640 medium.

2.4 *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-bottom microtiter plates (Nunc F96 microtiter plates, Denmark) were inoculated with 200 μl of each bacterial dilution series (from 10^2 to 10^6 CFU/ml). After 24 h of incubation at 37°C, 50 μl aliquots of MTS and various concentrations of PMS were added to each well so as to obtain final concentrations of 200 μg/ml for MTS and 100, 25, 6·25, 1·56, and 0·39 μM for PMS. The micro-

titer plates were then incubated for another 1 h, after which the OD at 490 nm was measured. This experiment was done in four replicates for each bacterial strain, and the data analysis were performed by linear regression analysis. Regression lines with the 95% confidence intervals were plotted for different levels of PMS and for each bacterial strain. The slopes and the r^2 of each regression line were recorded for the estimation of steepness of the line and the goodness of fit, respectively. An r^2 value of 1 indicates perfect correlation.

2.5 *Sep-Pak C_{18} purification*

The cell-free coelomic fluid of the earthworms (ECF) was diluted (vol/vol) with acidified water [0·1% (v/v) trifluoroacetic acid] and subjected to a Sep-Pak C_{18} cartridge (Waters Associates) equilibrated with 0·05% (v/v) trifluoroacetic acid. Elution was stepwise with 10, 20, 30, 40, 50, 60, 100% of acetonitrile. All fractions were concentrated by freeze-iced vacuum evaporation for later assay.

2.6 *Gel filtration chromatography*

The ECF was loaded onto a Sephadex G-10 column (1·5 × 60 cm) equilibrated with 0·02 M Tris-HCl (pH 8·0). Elution was with the same buffer at a flow rate of 1 ml/min and absorbances read at 280 nm. This chromatography was designed to remove pigments from the coelomic fluid, so, all fractions were pooled together, desalted and concentrated by ultrafiltration in a stirred cell system with a nominal molecular mass limit of 1 kDa (Filtron Technology Corporation, Northborough, MA, USA) against distilled water and lyophilized. After lyophilization, the sample was reconstituted in DPBS (pH 7·2) for later use.

2.7 *Anion exchange chromatography*

The reconstituted ECF pigment-free eluate (ECFPE) was loaded onto a DEAE-52 column (2·5 × 30 cm) equilibrated with DPBS (pH 7·2). Bound material was eluted in a linear gradient from 0 to 1·0 M NaCl in DPBS (pH 7·2) at a flow rate of 75 ml/h (total vol: 750 ml) and the effluent was monitored at 280 nm. The active fractions were pooled, concentrated by ultrafiltration with the same stirred cell system of 1 kDa mass limit, and lyophilized.

2.8 *Antimicrobial assay*

The antimicrobial activity test was made on bacterial suspensions in 96-well flat-bottom microtiter plates. Lyophilized ECFPE and other ECF effluents were reconstituted in DPBS (pH 7·2), respectively. The protein levels were determined for each eluate by the method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard. Protein concentrations for the ECFPE or other ECF effluents were adjusted to between 0·05 and 0·08 mg/ml by dilutions in the RPMI 1640 medium. Each well of 96-well microtiter plates was filled with 200 µl medium, 50 µl of each strain suspension, diluted 1 : 50 in the medium to obtain a final concentration of 10^4 cells/ml, and 30 µl of the ECFPE or other ECF effluents dilution as well. The growth control used 30 µl of the same volume of DPBS dilutions in the medium. Background OD was prepared with bacteria-free wells processed in the same way as the bacteria-containing wells. The plates were incubated for 24 h at 37°C with gentle stirring, and then the OD at 405 nm was measured for each well using the spectrophotometric quantification by a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). Afterwards, 20 µl of MTS-PMS mixture was added to each well for a further 1 h incubation. The OD of each well at 490 nm was measured for colorimetric quantification of soluble formazan production. To quantify the antimicrobial activity of each fraction, reconstituted lysozyme (10 mg/ml) was used as frame of reference. The relative OD for each well, based on measurements at 490 nm, was calculated (in percent) using the following equation: R = [(OD of antimicrobial dilution containing well – background OD)]/(OD of growth control well – background OD of growth control well)] × 100%, $A_s = R_l/R_s \times 100\%$; here R_l, R_s were the relative ODs for lysozyme and antimicrobial samples, respectively. The tests were carried out with four replicates in three independent experiments for each of the used bacteria, and the data analysis used the SPSS for Windows (Release 10·0·1, Chicago, Illinois, USA).

2.9 *Determination of minimal inhibitory concentrations of antimicrobial fractions*

Minimal inhibitory concentrations (MICs) of the isolated antimicrobial fractions against sensitive bacteria as described by Moore *et al* (1991) were improved. Lyophilized ECFPE or other ECF effluents were reconstituted in DPBS (pH 7·2) and the protein levels were determined for each fraction using the method described by Bradford (1976). By serial dilutions of the fractions in the 96-well microtiter plate the antimicrobial assay of each fraction was estimated under 405 or 490 nm measurements. The lowest concentration of each active fraction that showed any suppression of bacterial growth was defined as the minimal inhibitory concentration.

3. Results

3.1 *Quantitative assay of bacterial viability*

We tested the relationship between the MTS conversion and increasing inocula of test bacteria with 200 µg MTS

plus various concentrations of PMS after 1 h of exposure. The analysis of results showed a linear relationship between the OD and log CFU (figure 1, table 1). The slopes and the coefficients obtained by the regression analysis depended on the concentration of PMS used for each test bacterium. A relatively general pattern of MTS conversion rates increment with higher concentrations of PMS, i.e. generating higher slopes, can be observed for all six strains. However, at higher concentration of PMS as in the case of 100 μM, larger deviations from linearity were observed for some strains and very low slopes were obtained for *B. megaterium* and *S. aureus* (0·11 and 0·14, respectively) (table 1). Thus, a concentration of PMS with relatively higher conversion rates and more balanced linear relationships between formazan production and viable bacteria should be chosen as standardized parameters for the further antimicrobial activity assessments. In this study, the concentration of 25 μM PMS may be the best recommendation (table 1).

3.2 *Sep-Pak C_{18} purification*

We examined antimicrobial activity of the coelomic fluid against six test microorganisms of each fraction of a stepwise elution with 10%, 20%, 30%, 40%, 50%, 60% and 100% of acetonitrile, respectively. The results showed that the fraction eluted with 20% acetonitrile gave positive activity against *Arthrobacter* sp., *E. coli*, *S. aureus* (for *t* test, $P < 0{\cdot}01$) and less activity against *P. aeruginosa* ($0{\cdot}05 < P < 0{\cdot}01$) based on the colorimetric method (table 2). It performed similarly under spectrophotometric measurements at 405 nm except for results with *Arthrobacter* sp.. All the results based on both measurements at 405 and 490 nm were analysed by *t* test for statistically significant differences.

3.3 *Gel filtration chromatography*

The gel filtration chromatography for the ECF was designed to remove pigments from the coelomic fluid. The ECFPE under 405 nm measurements showed definite antimicrobial activity against *B. megaterium* and *P. aeruginosa* ($P < 0{\cdot}05$), and even stronger activity against *E. coli* and *S. aureus* ($P < 0{\cdot}01$) (table 3). It was also observed that the ECFPE had positive activity against *E. coli* ($P < 0{\cdot}01$) and *P. aeruginosa* ($P < 0{\cdot}05$) as estimated by

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

	Relationship between PMS concentration and bacteria inoculum (log CFU/ml)									
	100 μM		25 μM		6·25 μM		1·563 μM		0·39 μM	
Strain	Slope	r^2	Slope	r^2	Slope	r^2	Slope	r^2	Slope	r^2
A. hydrophila	0·38	0·963	0·21	0·827	0·24	0·491	0·10	0·338	0·07	0·788
S. aureus	0·14	0·723	0·46	0·818	0·38	0·805	0·34	0·713	0·26	0·787
P. aeruginosa	0·35	0·863	0·46	0·972	0·36	0·765	0·23	0·861	0·29	0·870
B. megaterium	0·11	0·747	0·28	0·981	0·11	0·909	0·26	0·761	0·24	0·885
Arthrobacter sp.	0·89	0·968	0·66	0·983	0·50	0·874	0·23	0·897	0·08	0·384
E. coli	0·18	0·959	0·37	0·891	0·12	0·795	0·17	0·920	0·10	0·851

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

Table 2. Antimicrobial activity quantitation of 20% acetonitrile eluate against sensitive strains.

					Sig. test[c] (*P* value)	
Species	R^a (405 nm)	*R* (490 nm)	A_s^b (405 nm)	A_s (490 nm)	405 nm	490 nm
E. coli 1·1543	29·4 ± 1·08	24·1 ± 1·09	23·5 ± 2·15	19·2 ± 1·29	0·002	0·006
P. aeruginosa 1·50	66·6 ± 0·77	62·1 ± 1·24	87·9 ± 2·34	113·5 ± 3·03	0·044	0·036
S. aureus 26001	50·7 ± 0·93	53·9 ± 1·05	32·7 ± 2·78	28·8 ± 0·21	0·001	0·001
Arthrobacter sp. 1·8	–[d]	43·2 ± 0·59	–	103·7 ± 3·30	–	0·008

[a]Relative ODs for each sensitive strain and also seen in context for details.
[b]Relative ODs of lysozyme activity divided by relative ODs of sensitive strains. Also seen in context.
[c]*T* test for statistically significant difference between each strain and its growth control. $a = 0{\cdot}05$ estimates significant antimicrobial activity. $a = 0{\cdot}01$ estimates the most significant activity.
[d]Antimicrobial activity not identified. Data are the means ± SE of quartet in triplicate experiments.

the colorimetric method at 490 nm, implying that no activity against *B. megaterium* or *S. aureus* similar to that at 405 nm occurred.

3.4 *Anion exchange chromatography*

The anion (DEAE-52) exchange chromatography was made with the reconstituted ECF pigment-free eluate. There was only one peak detected demonstrating antimicrobial activity against sensitive strains from the whole column eluates. This peak was collected at an effluent time of 1·62 h (volume: 122 ml) with a concentration of 0·162 M NaCl. The antimicrobial activity for this peak (termed peak 1) showed that it displayed resistance against *E. coli* ($P < 0·01$) and *P. aeruginosa* ($P < 0·05$) at 405 nm measurements (table 4). For comparison, the peak 1 also showed positive activity against *Arthrobacter* sp. ($P < 0·05$) and similar activities against *E. coli* and *P. aeruginosa* under 490 nm measurements.

Table 3. Results of the ECFPE against sensitive strains[a].

					Sig. test (*P* value)	
Species	*R* (405 nm)	*R* (490 nm)	A_s (405 nm)	A_s (490 nm)	405 nm	490 nm
B. megaterium 1·151	38·3 ± 2·81[b]	–	29·8 ± 1·14	–	0·016	–
P. aeruginosa 1·50	17·1 ± 1·01	21·8 ± 2·15	342·3 ± 9·65	323·3 ± 9·11	0·022	0·035
E. coli 1·1543	12·7 ± 0·38	8·74 ± 1·86	54·4 ± 3·80	52·9 ± 1·58	0·006	0·001
S. aureus 26001	4·64 ± 0·82	–	357·3 ± 3·00	–	0·001	–

[a]All items in table 3 and their corresponding computations are based on the protocol in table 2.
[b]Relative ODs corresponding to the measurements of the ECFPE.

Table 4. Results of anion exchange eluates of the ECFPE against sensitive strains[a].

					Sig. test (*P* value)	
Species	*R* (405 nm)	*R* (490 nm)	A_s (405 nm)	A_s (490 nm)	405 nm	490 nm
Arthrobacter sp. 1·8	–	41·1 ± 1·01	–	109·0 ± 3·72	0·025	0·013
E. coli 1·1543	14·7 ± 1·64	11·2 ± 0·81	47·0 ± 3·22	41·3 ± 2·49	0·004	0·000
P. aeruginosa 1·50	7·33 ± 0·96	5·45 ± 0·22	798·7 ± 40·6	1293·3 ± 89·8	0·030	0·039

[a]All items in table 4 and their corresponding computations are based on the protocol in table 2.

Table 5. Minimal inhibitory concentrations of isolated active fractions against sensitive bacteria.

	Minimal inhibitory concentrations (μg/ml)					
	AE[a]		ECFPE		Peak 1	
Microbes	405 nm	490 nm	405 nm	490 nm	405 nm	490 nm
Gram-positive						
S. aureus 26001	90	95	8	–[b]	–	–
B. megaterium 1·151	–	–	36	–	–	–
Arthrobacter sp. 1·8	–	50	–	–	–	50
Gram-negative	–					
E. coli 1·1543	40	25	18	10	25	20
P. aeruginosa 1·50	150	90	36	16	16	8

[a]AE stands for 20% acetonitrile eluate. [b]Antimicrobial activity not identified.
Minimal inhibitory concentrations were determined with serial dilutions of each active fraction in a 96-well microtiter plate. The lowest concentration of an active fraction that showed any suppression of bacterial growth was defined as MIC.

3.5 *Determination of minimal inhibitory concentrations*

With serial dilutions of the fractions in the 96-well microtiter plate, we examined the antimicrobial activity of each active fraction against sensitive strains for the MIC of each own. The ECFPE had the strongest activity against *E. coli* and *S. aureus* than other active fractions with the MICs of 18 and 8 μg/ml at 405 nm measurements, respectively (table 5). The peak 1 had the strongest activity against *P. aeruginosa* with MICs of 16 and 8 μg/ml using 405 and 490 nm measurements, respectively. The ECFPE was the only fraction tested that showed antimicrobial activity against *B. megaterium* by the spectrophotometric method.

4. Discussion

The MTS-PMS colorimetric assay was developed for distinguishing tiny differences between viable bacteria number of tests and controls by relating the bacteria dehydrogenase activity to the production of solubly coloured formazan. This approach, as it stands, can override defects possibly occurring in the antimicrobial assessments of inhibition-zone assays and colony-forming unit technique. To the inhibition-zone assays, effects as the thickness of agar culture, the permeability and diffusion of antimicrobial substances and the diameter measurement of inhibition-zones may mislead experiments even manipulated with delicate facitilities as a vernier caliper. To the colony-forming unit technique, the colony numbering always brings errors in. The MTS-PMS assay can, on the other hand, provide a solid and delicate rationale for the determination of antimicrobial activity by avoiding a lot of intermediate steps involved in the assessments.

To standardize the parameters involved in the MTS method, we investigated the relationship between the number of viable bacteria and the amount of MTS reduction with increasing inocula of test bacteria (10^2 to 10^6 CFU/ml) exposed to 200 μg/ml MTS and various concentrations of PMS (100 μM, 25 μM, 6·25 μM, 1·56 μM, and 0·39 μM). Among the different concentrations of PMS that were evaluated, the concentration of 25 μM showed relatively higher conversion rates (higher slopes) and more linear relationships between formazan production and viable bacteria for all test bacterial strains (figure 1, table 1). Therefore, we recommended this optimum for the determination of antimicrobial activity against test bacterial strains.

The ECF *E. f. andrei* and several of its column chromatography eluates contained some electro-stimulated substances (proteins) which were active against either Gram-positive or Gram-negative microorganisms. The antimicrobial activity existing in the ECF was suggested to arise from the yellow pigment (riboflavin), a lysozyme-like activity and fetidins (Hirigoyenberry *et al* 1991; Lassalle *et al* 1988). To avoid such effects of the yellow pigment (riboflavin), we used gel filtration chromatography to remove the yellow pigment from the ECF. As a result, the ECFPE showed relatively high antimicrobial activity against Gram-positive *B. megaterium*, *S. aureus* and Gram-negative *P. aeruginosa*, *E. coli* and no obvious activity against *A. hydrophila* (figure 2A). It was also the only reported activity against *B. megaterium* amongst the three isolated antimicrobial substances (proteins). Compared with lysozyme (figure 3A), the ECFPE displays had more than three times as much activity against *S. aureus* or *P. aeruginosa*, indicating some new component or combination rather than lysozyme-like activity alone. For effects on *E. coli* and *P. aeruginosa*, the MICs of the ECFPE at 490 nm measurements were lower than those at 405 nm measurements, indicating that the colorimetric method may be more sensible than the spectrophotometric method in the antimicrobial activity detection.

The Sep-Pak C_{18}-eluted ECF with 20% acetonitrile had a similar antimicrobial spectrum to the ECFPE except against Gram-positive *Arthrobacter* sp. rather than *B. megaterium*. The ECF effluent with 20% acetonitrile had less antimicrobial activity against sensitive bacteria by contrast with the ECFPE. Milochau *et al* (1997) reported that the antimicrobial activity of dialysed coelomic fluid of the lumbricid *E. f. andrei* was 25% less than that of the coelomic fluid. They contributed this decrease to the partial elimination of riboflavin and lysozyme after dialysis (Milochau *et al* 1997). We consider that the ECF would lose most of riboflavin and most of the lysozyme after the Sep-Pak C_{18} elution. Thus, the high relative antimicrobial activity of the 20% acetonitrile eluate against *Arthrobacter* sp. or *P. aeruginosa* (at 103·7 or 113·5 of A_s at 490 nm) provides a demonstration of the existence of a novel activity different from that of the original lysozyme. The good correlations between *R* values of 20% acetonitrile eluate (AE) at 405 nm and 490 nm gave further assurance for the antimicrobial activity (figure 2).

The anion (DEAE-52) exchange eluate (peak 1) of the ECFPE had the strongest activity against *P. aeruginosa* amongst the three isolated active fractions with A_s value up to 1293·3 at 490 nm measurements (figure 3B). *Pseudomonas aeruginosa* is a refractory pathogen causing lethal or chronic diseases such as *P. aeruginosa* bacteraemia, urinary tract infection (UTI) and chronic lung infection, primarily due to development of resistance resulting from its polysaccharide matrix-based biofilms. A recent study (Turnidge *et al* 2002) showed that amikacin ranked most potent with 91·5% susceptibility, and the susceptibility to quinolones for the *P. aeruginosa* isolates was only 63·2–67·0%. The relative OD of peak 1 against *P. aeruginosa* at 490 nm is 5·45 ± 0·22 (SE), equivalent to 94·6%

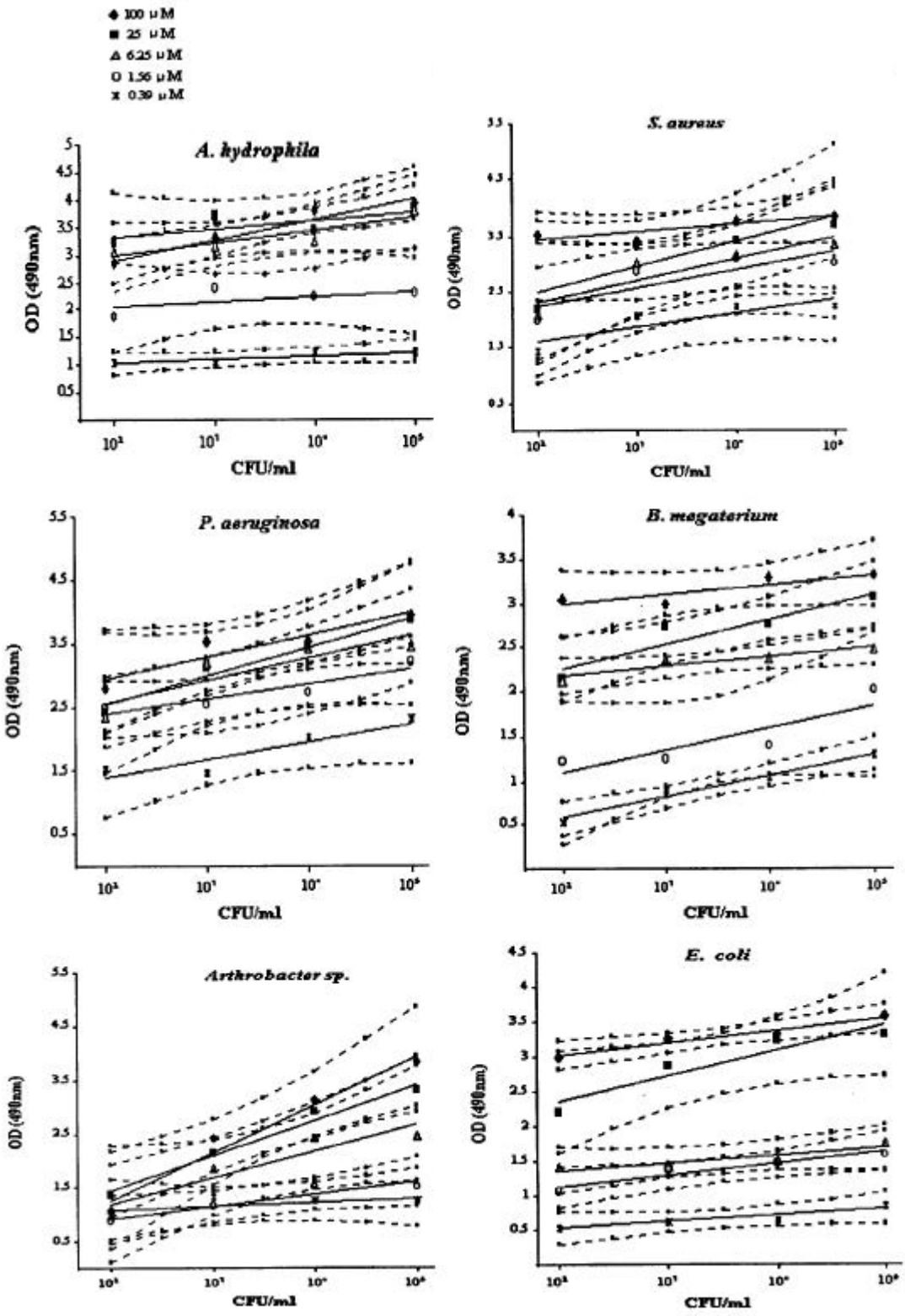

Figure 1. Relationship between the amount of MTS and increasing inocula of each test bacterial strain. The solid lines were obtained by linear regression analysis, and the symbols stand for the means of the triplicates. Dotted lines represent the 95% confidence intervals of the regression lines. See the text for details.

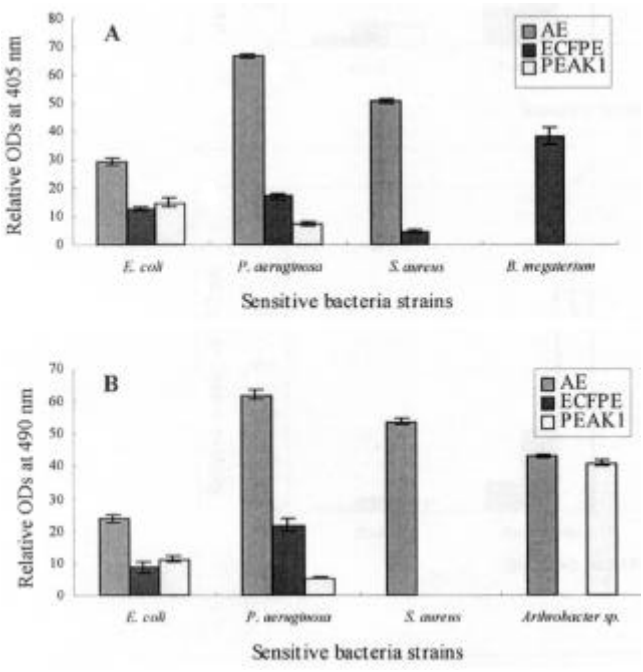

Figure 2. Relative ODs of isolated active fractions against sensitive bacteria strains at (**A**) 405 nm and (**B**) 490 nm. AE stands for 20% acetonitrile eluate. ECFPE stands for pigment-free eluate of coelomic fluid of the earthworm. Peak 1 comes from one of anion exchange eluates of the ECFPE.

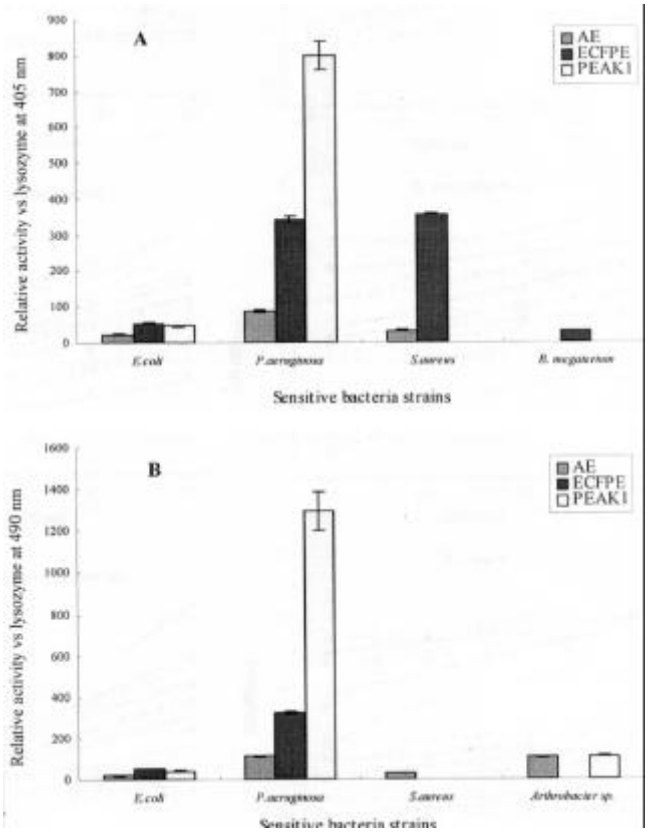

Figure 3. Relative activity of isolated active fractions vs lysozyme against sensitive bacteria strains at (**A**) 405 nm and (**B**) 490 nm. See the text for details.

susceptibility, predicting potential as an anti-pseudomonal candidate. However, the antimicrobial activity of the ECFPE against *S. aureus* or *B. megaterium*, which was detected originally at 405 nm measurements, did not recur in the 490 nm measurements. The AE and peak 1 were identified as relatively high activities against *Arthrobacter* sp. only by colorimetric assays (figure 2B).

In conclusion, our data showed that the MTS-PMS colorimetric assay can be used to evaluate the antimicrobial activity of purified active fractions from natural resources. Compared with other methods commonly used in this field, the MTS-PMS assay excels with more delicacy, more swiftness, and more conveniency. By this method, we reconfirmed several novel antimicrobial activities occurring in the coelomic fluid of the earthworm *E. f. andrei* and made quantitative comparisons of each activity. During the time, we also optimized the parameters for the system which may be used in the assessments of antimicrobial activity against bacteria. Although the work in this study is limited to routine chromatography techniques, further research may recur to high-performance liquid chromatography (HPLC) for screening out finer antimicrobial components from the existing activities.

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