SUPPLEMENTARY MATERIAL

An insight into antimicrobial activity of the freshwater bryozoan *Pectinatella magnifica*

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Abstract

The antimicrobial activity of five crude extracts of the freshwater bryozoan *Pectinatella magnifica* (Leidy, 1851) was evaluated *in vitro* for the first time. *P. magnifica* acetone extract exhibited the highest antibacterial activity (MIC 0.004-0.350 mg/mL and MBC 0.007-0.500 mg/mL), while its methanol extract showed the most promising antifungal activity (MIC 0.03-0.12 mg/mL and MFC 0.06-0.25 mg/mL). Furthermore, at a concentration of 0.25 MIC, the methanol extract reduced biofilm formation of the bacterial strain *Pseudomonas aeruginosa* PAO1 in a considerable extent (59.14%). FTIR spectra of the most active extracts indicate the presence of carbonyl compounds, long-chain alcohols and/or sterols. According to the experimental data obtained, *P. magnifica* methanol extract may be considered as a good resource of novel natural products with potent anti-biofilm activity against the bacterium well known for its resistance.

Keywords: Bryozoa; extracts; anti-biofilm activity, *Pseudomonas aeruginosa* PAO1

Experimental

Biological material

The sample of *Pectinatella magnifica* (Leidy, 1851) was collected in River Danube (Vojvodina, Republic of Serbia) in October 2013. Voucher specimen has been deposited in the Zoology Collection of the Department of Biology and Ecology of the University of Novi Sad, Republic of Serbia (BRY 004).

Extraction

The dried parts of *P. magnifica* were ground (4 g) and extracted thrice with water, methanol, ethyl acetate, acetone and ether for 1 h at room temperature, respectively. Then the extracts were evaporated to dryness and stored at –20 °C until further use.

FTIR analysis

Fourier-transform infrared (FTIR) spectra of the most active *P. magnifica* extracts were recorded in the attenuated total reflection mode (ATR) using a Nicolet 6700 FTIR Spectrometer (Thermo Scientific).

Determination of antibacterial activity

The following Gram-positive (*Staphylococcus aureus* ATCC 6538, *Bacillus cereus* clinical isolate, *Micrococcus flavus* ATCC 10240 and *Listeria monocytogenes* NCTC 7973) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Escherichia coli* ATCC 35210 and *Enterobacter cloacae* human isolate) were used. The antibacterial assay was carried out by a microdilution method (CLSI 2009, Tsukatani et al. 2012). The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. The bryozoan extracts were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and immediately added in Tryptic Soy broth (TSB) medium (100 µL) with bacterial inoculum (1.0×10^4 CFU per well). The lowest concentrations without visible growth (at the

binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MICs obtained from the susceptibility testing of various bacteria to the extracts tested were determined also by a colorimetric microbial viability assay based on reduction of INT ((*p*-iodonitrotetrazolium violet) [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) color and compared with the positive control for each bacterial strain. The MBCs were determined by serial sub-cultivation of 2 μ L into microtiter plates containing 100 μ L of broth per well and further incubation for 24 hours. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank (broth medium plus diluted extracts) and the positive control. Streptomycin (Sigma-Aldrich S6501) and ampicillin (Sigma-Aldrich A9393) were used as positive controls (1 mg/mL in sterile physiological saline), while solution of 5% DMSO was used as a negative control. All experiments were performed in duplicate and repeated three times.

Determination of antifungal activity

As far as the antifungal bioassay is concerned, eight fungi (*Aspergillus fumigatus* human isolate, *A. versicolor* ATCC 11730, *A. ochraceus* ATCC 12066, *A. niger* ATCC 6275, *Trichoderma viride* IAM 5061, *Penicillium funiculosum* ATCC 36839, *P. ochrochloron* ATCC 9112 and *P. verrucosum* var. *cyclopium* food isolate) were used. In order to investigate the antifungal activity of the bryozoan extracts a modified microdilution technique was applied (Hanel & Raether 1998; Espaniel-Ingroff 2001). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v) and spore suspension was adjusted with sterile saline to a concentration of 1.0×10^5 . *P. magnifica* extracts were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and immediately added in broth Malt medium with inoculum. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μ L of the extracts dissolved in medium and incubated for 72 hours at 28°C. The

lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. The commercial fungicides bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Sabac, Serbia) were applied as positive controls (1-3000 µg/mL), while solution of 5% DMSO was used as a negative control. All experiments were performed in duplicate and repeated three times.

Bacterial strain, growth media and culture conditions

The strain *Pseudomonas aeruginosa* PA01 (ATCC 27853) belongs to the collection of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Sinisa Stankovic" (IBISS), University of Belgrade, Belgrade, Serbia. Bacteria were routinely grown in Luria-Bertani (LB) medium (1% w/v NaCl, 1% w/v Tryptone, 0.5% w/v yeast extract) with shaking (220 rpm) and cultured at 37 °C.

Anti-biofilm activity

The effect of different concentrations of methanol and acetone extracts of *P. magnifica* (ranging from 0.5, 0.25 and 0.125 of MIC; MIC was 0.06 and 0.0075 mg/mL, respectively) on biofilm forming ability was tested on polystyrene flat-bottomed microtitre 96 well plates as described by Spoering & Lewis (2001) and Drenkard & Ausubel (2002), with some modifications. Briefly, 100 µL of overnight culture of *P. aeruginosa* (inoculum size was 1×10^8 CFU/mL) was added to each well of the plates in the presence of 100 µL subinhibitory concentrations (subMIC) of the extracts (0.5, 0.25 and 0.125 MIC) or 100 µL medium (control). After incubation for 24 h at 37 °C, each well was washed twice with sterile PBS (pH 7.4), dried, stained for 10 min with 0.1% crystal violet in order to determine the biofilm mass. After drying, 200 µL of 95% ethanol (v/v) was added to solubilise the dye that had stained the biofilm cells. The excess stain was washed off with dH₂O. After 10 min, the content of the wells was homogenized and the absorbance at $\lambda = 625$ nm was read on a Sunrise™ - Tecan ELISA reader. The experiment was done in triplicate and repeated two times and values were presented as mean values $\pm$ SE.

Anti-twitching and flagella motility activity

After growth in the presence or absence of the methanol and acetone extracts (subMIC, 0.5 MIC), streptomycin and ampicillin (subMIC), the cells of *P. aeruginosa* PA01 were washed twice with sterile PBS and resuspended in PBS at 1×10^8 CFU/mL (OD of 0.1 at 660 nm). Briefly, cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37 °C. Plates were then removed from the incubator and incubated at room temperature for two more days. Colony edges and the zone of motility were measured with a light microscope (O'Toole & Kolter 1998a,b). SubMIC (0.5 MIC, in range from 0.030000 to 0.000375 mg/mL) of the extracts were mixed into 10 mL of molten MH medium and poured immediately over the surface of a solidified LBA plate as an overlay. The plate was point inoculated with an overnight culture of PA01 once the overlaid agar had solidified and incubated at 37 °C for 3 days. The extent of swimming was determined by measuring the area of the colony (Sandy & Foong-Yee 2012). The experiment was done in triplicate and repeated two times. The colony diameters were measured three times in different direction, while the values are presented as mean values $\pm$ SE.

Inhibition of pyocyanin production

Overnight culture of *P. aeruginosa* PA01 was diluted to OD₆₀₀ nm 0.2. Then, the methanol and acetone extracts (0.030000-0.000375 mg/mL, respectively) was added to *P. aeruginosa* (5 mL) and incubated at 37 °C for 24 h. The treated culture was extracted with chloroform (3 mL), followed by mixing the chloroform layer with 0.2 M HCl (1 mL). Absorbance of the extracted organic layer was measured at 520 nm using a Shimadzu UV1601 spectrophotometer (Kyoto, Japan) (Sandy & Foong-Yee 2012). The experiment was done in triplicate and repeated two times. The experiment was done in triplicate and repeated two times; the values obtained /expressed as ratio (OD₅₂₀/OD₆₀₀) $\times$ 100/ are presented as mean values $\pm$ SE.

Data analysis

The results (expressed as mean values $\pm$ standard errors) were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$ (SPSS v. 18.0 program).

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Samples	Biofilm formation (%)*		
	0.5 MIC	0.25 MIC	0.125 MIC
Methanol extract	45.18	40.86	–
Acetone extract	–	–	–
Streptomycin	74.20	73.61	64.14
Ampicillin	86.82	90.30	42.73

Table S3. Effects of selected extracts of *Pectinatella magnifica* on biofilm formation of *Pseudomonas aeruginosa* PAO1.

* Biofilm formation values were calculated as: (mean A₆₂₀ treated well)/(mean A₆₂₀ control well)×100.
 Values are expressed as means ± SE.
 – no activity

Table S4. Twitching and motility activity of of selected extracts of *Pectinatella magnifica*.

Samples	Colony diameter (mm ± SD)	Colony color	Protrusions diameter (µm)	Colony edge on microscope
Methanol extract	11.30±2.08	white	56-96	slightly reduced protrusions
Acetone extract	14.33±3.06	white	–	reduced protrusions
Streptomycin	7.67±0.58	white	–	reduced protrusions
Ampicillin	8.00±0.00	white	64-160	slightly reduced protrusions
Control (PAO1)	21.00±3.60	green	80-240	regular protrusions

Table S5. Effects of selected extracts of *Pectinatella magnifica* on the production of pyocyanin by *Pseudomonas aeruginosa* PAO1.

Samples	Pyocyanin (%±SE)
Methanol extract	94.96±1.23
Acetone extract	74.43±1.15
Streptomycin	48.02±1.29
Ampicillin	44.15±1.05

Control (PA01)	107.90 ± 1.07
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