SUPPLEMENTARY MATERIAL

***In vitro* anti-biofilm activity of the freshwater bryozoan *Hyalinella punctata*: a case study of *Pseudomonas aeruginosa* PAO1**

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Abstract

The anti-biofilm and possible anti-quorum sensing effects against the strain *Pseudomonas aeruginosa* PAO1 of five crude extracts of the freshwater bryozoan *Hyalinella punctata* (Hancock, 1850) was evaluated *in vitro* for the first time. *H. punctata* ethyl acetate extract (HpEtAc) exhibited the highest anti-biofilm activity reducing the biofilm formation of *P. aeruginosa* PAO1 in the range of 80.63-88.13%. While all tested extracts reduced the twitching motility of the aforementioned bacterial strain, HpEtAc showed to be the most effective. Finally, at a concentration of 0.5 MIC, the same extract mostly inhibited the production of pyocyanin by *P. aeruginosa* PAO1 (71.53%). In comparison both with the positive controls used (streptomycin and ampicillin, 67.13 and 69.77%, respectively), HpEtAc was found to inhibit pyocyanin in a higher extent. An extensive chemical characterisation of this particular extract may result in isolation and identification of novel lead compounds targetting *P. aeruginosa*, an opportunistic human pathogen.

Keywords: Bryozoa; extracts; anti-biofilm activity; anti-quorum sensing activity

Experimental

Biological material

The sample of *Hyalinella punctata* (Hancock, 1850) was collected in River Sava (Belgrade, 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 003).

Extraction

The dried parts of *H. punctata* were ground (4 g) and extracted thrice with water, methanol, ethyl acetate, acetone and hexane 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) spectrum of the most active *H. punctata* extract (ethyl-acetate) was recorded in the attenuated total reflection mode (ATR) using a Nicolet 6700 FTIR Spectrometer (Thermo Scientific).

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 tested extracts of *H. punctata* (ranging from 0.5, 0.25 and 0.125 of MIC; MICs were ranging from 0.06 to 0.18 mg/mL) 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 μ L of overnight culture of *H. punctata* (inoculum size was 1×10^8 CFU/mL) was added to each well of the plates in the presence of 100 μ L subinhibitory concentrations (subMIC) of the extracts (0.5, 0.25 and 0.125 MIC) or 100 μ L 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 μ L 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 tested extracts (subMIC, 0.5 MIC), streptomycin and ampicillin (subMIC), the cells of *P. aeruginosa* PAO1 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 of 0.03-0.09 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 PAO1 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 OD_{600 nm} 0.2. Then, tested extracts (0.03-0.09 mg/mL, respectively) were added to *P. aeruginosa* respectively (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)×100/ are presented as mean values ± SE.

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Figure S1. Light microscopy of colony edges of *Pseudomonas aeruginosa* PAO1 in twitching motility plates, grown in the presence or absence of tested extracts of *Hyalinella punctata*.

The bacterial colonies grown with tested extracts at a concentration of 0.5 MIC (**A-E**) were rounded, having a smooth domed shape and lacking a hazy zone surrounding the colonies; in the absence of tested extracts, *Pseudomonas aeruginosa* PAO1 produced flat, widely spread, irregularly shaped colonies (**H**); in the presence of streptomycin (0.5 MIC), *P. aeruginosa* colonies had reduced protrusions (**F**); in the presence of ampicillin, *P. aeruginosa* colonies had slightly reduced protrusions (**G**); Magnification: (**A-H**) $\times 100$.

Table S2. Twitching and motility activity of tested extracts of *Hyalinella punctata*.

Samples	Colony diameter (mm $\pm$ SD)	Colony color	Protrusions diameter (μ m)	Colony edge on microscope
Methanol extract	18.33 $\pm$ 2.52	green	24-120	slightly reduced protrusions
Acetone extract	15.33 $\pm$ 5.03	light green	40-48	slightly reduced protrusions
Hexane extract	18.67 $\pm$ 5.51	white	–	reduced protrusions
Ethyl acetate extract	12.67 $\pm$ 3.79	light green	–	reduced protrusions
Aqueous extract	17.67 $\pm$ 3.21	white	56-80	slightly reduced protrusions
Ampicillin	8.00 $\pm$ 0.00	white	64-160	slightly reduced protrusions
Streptomycin	7.67 $\pm$ 0.58	white	–	reduced protrusions
Control (PAO1)	22.33 $\pm$ 3.60	green	80-240	regular protrusions

Table S4.
inhibitory (MIC)
(MBC)

punctata extracts.

Samples	<i>Pseudomonas aeruginosa</i> (MIC/MBC±SE)*
Methanol extract	0.060±0.004 ^a 0.120±0.000 ^a
Acetone extract	0.090±0.010 ^b 0.120±0.007 ^a
Hexane extract	0.120±0.007 ^c 0.250±0.020 ^b
Ethyl acetate extract	0.090±0.007 ^b 0.120±0.000 ^a
Aqueous extract	0.180±0.000 ^d 0.250±0.010 ^b
Ampicillin	0.740±0.003 ^c 1.240±0.003 ^d
Streptomycin	0.170±0.007 ^d 0.340±0.003 ^c

Minimum
and bacterial
concentrations of
Hyalinella

* MIC/MBC (mg/mL)

Indicated letters mean significant differences (p<0.05).