SUPPLEMENTARY MATERIAL

An insight into anti-biofilm and anti-quorum sensing activities of the selected anthocyanidins: the case study of *Pseudomonas aeruginosa* PAO1

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Abstract

Anti-biofilm activity of three anthocyanidins (pelargonidin, cyanidin and delphinidin) was evaluated for the first time at *in vitro* conditions. All the compounds reduced the formation of *Pseudomonas aeruginosa* PAO1 biofilm at low subMIC (0.125 MIC) with delphinidin (c 56.25 µg/mL) being the most active (43%). In comparison, ampicillin (c 93.75 µg/mL) and streptomycin (c 21.25 µg/mL) (used as positive controls) were considerably less effective at the same subMIC (8 and 12%, respectively). Furthermore, at 0.5 MIC (c 225 µg/mL) this anthocyanidin molecule partly reduced the bacterial protrusions. However, no any of the aforementioned compounds inhibited the production of pyocyanin by the bacterial strain *P. aeruginosa* PAO1. Taken all together, the delphinidin scaffold could be taken into consideration for the design of the novel and more effective anti-biofilm agents inspired by the anthocyanidins.

Keywords: flavanone-7-O-glucoside; delphinidin; infectious diseases; bacterial resistance; anti-biofilm activity; low sub-MIC

Experimental

Bacterial strain, growth media and culture conditions

Pseudomonas aeruginosa PA01 (ATCC 27853) used in this study originated from the collection of the Mycoteca, Institute for Biological Research "Sinisa Stankovic" (IBISS), 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.

Antibacterial activity

Selected anthocyanidin compounds purchased from Sigma-Aldrich (Munich, Germany) were tested. The antibacterial assay was performed as previously described (Tsukatani et al. 2012; Pejin, Iodice et al. 2014). Streptomycin (Sigma-Aldrich, Munich, Germany) and ampicillin (Panfarma, Belgrade, Serbia) (1 mg/mL in sterile physiological saline) were applied as positive controls, while 5% DMSO (Sigma-Aldrich, Munich, Germany) was applied as a negative control. A number of wells were reserved in each plate for control of sterility (no inoculum added), inoculum viability (no compounds added) and 5% DMSO inhibitory effect.

Anti-biofilm activity

The effect of three different subinhibitory concentrations (subMICs) of selected anthocyanidins (ranging from 0.5 to 0.125 of MIC) on biofilm forming ability was tested on polystyrene flat-bottomed microtitre 96 well plates, as described previously with some modifications (Spoering & Lewis 2001; Drenkard & Ausubel 2002; Pejin, Savic et al. 2014). 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 subMICs of the compounds tested 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 homogenised 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. The values obtained are presented as mean values $\pm$ SE.

Anti-twitching and flagella motility activity

After growth in the presence or absence of the selected anthocyanidin compounds (subMIC; 0.5 MIC), streptomycin and ampicillin (0.5 MIC), 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). SubMICs of the compounds tested 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 colony diameters were measured three times in different direction. The experiment was done in triplicate and repeated two times. The values obtained are presented as mean values $\pm$ SE.

Pyocyanin production inhibitory activity

Overnight culture of *P. aeruginosa* PA01 was diluted to OD_{600 nm} 0.2. Then, the compounds tested (subMIC, 0.5 MIC), 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 values were expressed as ratio (OD₅₂₀/OD₆₀₀) $\times$ 100. The experiment was done in triplicate and repeated two times. The values obtained are presented as mean values $\pm$ SE.

Statistical analysis

All assays were carried out in triplicate. The results expressed as mean values with standard errors were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test ($\alpha = 0.05$). This analysis was carried out using SPSS v. 18.0 program.

References

Drenkard E, Ausubel FM. 2002. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature*. 416:740–743.

O'Toole GA, Kolter R. 1998a. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol*. 28:449–461.

O'Toole GA, Kolter R. 1998b. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol*. 30:295–304.

Pejin B, Iodice C, Tommonaro G, Stanimirovic B, Ciric A, Glamoclija J, Nikolic M, De Rosa S, Sokovic M. 2014. Further *in vitro* evaluation of antimicrobial activity of the marine sesquiterpene hydroquinone avarol. *Curr Pharm Biotechnol*. 15:583–588.

Pejin B, Savic A, Sokovic M, Glamoclija J, Ciric A, Nikolic M, Radotic K, Mojovic M. 2014. Further *in vitro* evaluation of antiradical and antimicrobial activities of phytol. *Nat Prod Res*. 28:372–376.

Sandy SM, Foong-Yee T. 2012. Anti-quorum sensing and antimicrobial activities of some traditional Chinese medicinal plants commonly used in South-East Asia. *Malaysian J Microb*. 8:11–20.

Spoering AL, Lewis K. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing of antimicrobials. *Bacteriol.* 183:6746–6751.

Tsukatani T, Suenaga H, Shiga M, Noguchi K, Ishiyama M, Ezoe T, Matsumoto K. 2012. Comparison of WST-8 colorimetric method and CLSI broth microdilution method for susceptibility testing against resistant bacteria. *J Microbiol Methods.* 90:160–166.

Table S1. Minimum inhibitory (MIC) and bactericidal (MBC) concentrations of selected anthocyanidins.

Anthocyanidins	<i>Pseudomonas</i>
	<i>aeruginosa</i> PAO1
	MIC/MBC
	(mg/mL)
Delphinidin	0.450
	0.900
Pelargonidin	0.450
	0.900
Cyanidin	1.000
	1.350
Streptomycin	0.170
	0.370
Ampicillin	0.750
	1.350

Table S2. Effects of selected anthocyanidins on twitching and flagella motility activity of *Pseudomonas aeruginosa* PAO1.

Flavonoids	Colony diameter (mm±SD)	Colony colour	Protrusions diameter (µm)	Colony edge on microscope
Delphinidin	9.33±0.58	light green	48-120	partly reduced protrusions
Pelargonidin	8.33±0.93	light green	40-80	slightly reduced protrusions
Cyanidin	9.00±1.00	white	24-80	slightly reduced protrusions
Ampicillin	9.33±0.58	light green	40-104	partly reduced protrusions
Streptomycin	8.33± 0.58	white	40-96	slightly reduced protrusions
Control (PAO1)	10.33±0.58	green	40-160	regular protrusions

Table S3. Effects of selected anthocyanidins on the production of pyocyanin by *Pseudomonas aeruginosa* PAO1.

Anthocyanidins	Pyocyanin (%±SE)
Delphinidin	59.29±1.03
Pelargonidin	98.87±1.14
Cyanidin	57.68±1.09
Ampicillin	40.04±0.98
Streptomycin	22.41±1.11
Control (PAO1)	51.33±1.07