

1 The use of quorum sensing inhibitors to interfere with biofilm formation and development in  
2 *Burkholderia multivorans* and *Burkholderia cenocepacia*.

3

4 Gilles Brackman<sup>a</sup>, Ulrik Hillaert<sup>b</sup>, Serge Van Calenbergh<sup>b</sup>, Hans J Nelis<sup>a</sup>, Tom Coenye<sup>b\*</sup>

5 a Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, B-9000  
6 Ghent, Belgium.

7 b Laboratory of Medicinal Chemistry, Ghent University, Harelbekestraat 72, B-9000 Ghent,  
8 Belgium.

9

10 [gilles.brackman@ugent.be](mailto:gilles.brackman@ugent.be)

11 [ulrik.hillaert@ugent.be](mailto:ulrik.hillaert@ugent.be)

12 [serge.vancalenbergh@ugent.be](mailto:serge.vancalenbergh@ugent.be)

13 [hans.nelis@ugent.be](mailto:hans.nelis@ugent.be)

14 [tom.coenye@ugent.be](mailto:tom.coenye@ugent.be) \*Correspondence and reprints

15

16

17

18

19

20

21

22

23

24

25

1 **Abstract:**

2 *Burkholderia cepacia* complex strains are opportunistic pathogens, causing life-threatening  
3 infections in CF patients. *Burkholderia cepacia* complex strains are resistant to many  
4 antimicrobial agents and commonly produce biofilms *in vitro* and *in vivo*. This contributes to  
5 their virulence and makes *Burkholderia* infections difficult to treat. Recently, the quorum  
6 sensing system of *Burkholderia* spp. has been found to affect their biofilm forming ability,  
7 making it an attractive target for antimicrobial therapy. However, detailed information about  
8 the antibiofilm effect of these compounds is still lacking. In the present study we evaluated  
9 the anti-biofilm effect of several known quorum sensing inhibitors. The effect on  
10 *Burkholderia* spp. biofilm formation was examined using crystal violet, resazurin and SYTO9  
11 staining, confocal laser scanning microscopy as well as plating. Used in sub-inhibitory  
12 concentrations, several compounds interfered with biofilm formation by *Burkholderia* spp.  
13 Our results suggest that the quorum sensing inhibitors affect later stages of biofilm formation  
14 and detachment.

15

16 Keywords: *Burkholderia cepacia* complex; biofilms; quorum sensing

17

18

19

20

21

22

23

24

25

## 1 **1. Introduction**

2 *The Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacteria  
3 [6]. Bcc species are opportunistic pathogens causing severe infections in patients with  
4 immune suppression, chronic granulomatous disease and cystic fibrosis (CF) [21]. Although  
5 all Bcc species are found in CF patients, overall *Burkholderia multivorans* and *Burkholderia*  
6 *cenocepacia* predominate [20; 38]. Bcc species are capable of forming biofilms *in vitro* and *in*  
7 *vivo* [8], which probably plays an important role in virulence [35]. In addition, bacteria  
8 growing in biofilms are less susceptible to many antibacterial agents [5]. For these reasons,  
9 novel approaches to treat these biofilm-related infections are needed. A possible novel target  
10 is the bacterial communication system [37]. Bacteria monitor their population density through  
11 the production and sensing of small signal molecules called autoinducers [3]. This  
12 communication system, known as quorum sensing (QS), consists of three components: signal  
13 molecules, signal synthases and signal receptors. At low population density only basal  
14 amounts of diffusible signal molecules are produced, not provoking an effect. However, when  
15 the population density is sufficiently high, signal molecules will bind to a receptor and this  
16 signal/receptor complex will then induce or repress the transcription of QS regulated genes.  
17 Several N-acyl-homoserine-lactone (AHL)-dependent QS systems have been reported in  
18 *Burkholderia* species [37]. In most Bcc species, CepI catalyzes the production of two AHL's,  
19 N-octanoyl-homoserine lactone (C8-HSL) (in high amounts) and N-hexanoyl-homoserine  
20 lactone (C6-HSL) (in lower amounts). *B. cenocepacia* epidemic strains contain a second QS  
21 system, CciIR [2]. The major signal molecule involved in the latter is C6-HSL [22]. In some  
22 *Burkholderia* spp. a quinolone dependent QS system can also be found [42]. This type of  
23 communication system was previously described in *Pseudomonas aeruginosa* and makes use  
24 of hydroxy-alkylquinolines (HAQ) as signal molecules [30]. It has recently been discovered  
25 that HAQs produced by *Burkholderia* spp. predominantly contain an unsaturated aliphatic

1 side chain and are typically methylated [42]. In several *Burkholderia* spp., the QS system is  
2 presumably involved in biofilm formation [8; 14; 41]. Hence, QS inhibitors have been  
3 proposed as potential novel antibiofilm agents [37]. Since these QS inhibitors are typically  
4 used in concentrations below the minimal inhibitory concentration (MIC), it is less likely that  
5 they would impose a selective pressure for the development of resistance. Many studies have  
6 been conducted to find natural and synthetic QS inhibitors [9-10; 15-16; 18; 26; 31-32; 34;  
7 40; 44]. However, detailed information about the antibiofilm effect of these compounds is still  
8 lacking. In the present study, we examined the antibiofilm effect of several established QS  
9 inhibitors in *B. multivorans* and *B. cenocepacia*, the two Bcc species most often responsible  
10 for infections in CF patients.

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

## 1 2. Materials and Methods

### 2 2.1. QS inhibitors

3 Cinnamaldehyde [26], resveratrol [43], L-canavanine [16], 4-nitropyridine N-oxide, p-  
4 benzoquinon and indole [31], azithromycin [40], ceftazidime hydrate and tobramycin [10],  
5 farnesol [9], (-)- epigallocatechin gallate and (+)- catechin hydrate [15], 2-amino-4-  
6 chlorobenzoic acid (4CABA) , 2-amino-6-chlorobenzoic acid (6CABA) and 2-amino-6-  
7 fluorobenzoic acid (6FABA) [18], curcumin [34], baicalein, baicalin hydrate and esculin  
8 hydrate [44] were purchased from Sigma-Aldrich (Bornem, Belgium). Esculetin [44] and  
9 N<sup>3</sup>-(2-thienylcarbonyl)-4-bromo-1,5-dimethyl-1H-pyrazole-3-carbohydrazide (“compound  
10 1”) [32] were purchased from Acros Organics (Geel, Belgium). N<sup>7</sup>-(6-tert-butyl-2,3-dihydro-  
11 2-methylpyridazin-4-yl)-5-chlorothiophene-2-carbohydrazide (“compound 3”) was  
12 synthesized as previously described [32]. All compounds were diluted in 0.5 % DMSO. A  
13 control solution (CS) containing only DMSO was made in MilliQ water. The stock solutions  
14 were stored at -20°C.

15

### 16 2.2. Strains and culture conditions

17 *Chromobacterium violaceum* CV026 [24] and *Escherichia coli* JB523 [1] were grown  
18 aerobically on Luria Bertani (LB) agar (BD, Sparks, MD, USA) containing 20 µg/ml  
19 kanamycin or 100 µg/ml tetracycline, respectively. For *E. coli* JB523 the medium contained 4  
20 % NaCl. The QS-inhibition (QSI) selector *P. aeruginosa* QSI2 [31] was cultured aerobically  
21 in ABT minimal medium (AB medium, containing 2.5 mg/l of thiamine) supplemented with  
22 0.5 % glucose, 0.5 % casamino acids and 80 µg/ml gentamycin. *B. cenocepacia* strains  
23 LMG18828 and LMG16656 (containing a CepI/R and CciI/R QS system) and *B. multivorans*  
24 strains LMG13010 and LMG17588 (containing the CepI/R QS system) were cultured  
25 aerobically on Tryptone Soya Agar (Oxoid, Hampshire, UK) or in Scientific Cystic Fibrosis

1 Medium (SCFM) [28]. All strains were grown at 37°C, with the exception of *E. coli* JB523  
2 (30°C) and *C. violaceum* CV026 (27°C).

3

#### 4 2.3. Determination of the minimal inhibitory concentration (MIC)

5 MIC's were determined using a modified microdilution assay in 96-well microtiter plates  
6 (MTP), using the media mentioned above [13]. The inoculum, prepared from a 24 h old  
7 culture contained approximately  $10^6$  CFU/ml in double concentrated medium. The  
8 compounds stock solutions were diluted in MilliQ water in a series of twelve 2-fold dilutions  
9 in the wells of a 96 well microtiter plate (MTP) (TPP, Trasadingen, Switzerland). Two rows  
10 of dilutions were made for each compound and the volume of each sample was 100  $\mu$ l. To the  
11 wells of one row of each compound, 100  $\mu$ l inoculum was added. To the other row, 100  $\mu$ l  
12 double concentrated medium was added as a negative control. The plates were incubated for  
13 24 h and the absorbance at 590 nm was measured using a Victor Wallac<sup>2</sup> multilabel counter  
14 (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). The absorbances of the  
15 negative controls were subtracted from those of the corresponding inoculated wells to give net  
16 absorbances. The plates were incubated for 24 h. For all further experiments, a single sub-  
17 MIC concentration was selected for each compound, being the highest concentration that did  
18 not affect growth in any of the strains included in the present study.

19

#### 20 2.4. QS inhibition (QSI) assays

21 *C. violaceum* CV026 is a mini-Tn5 mutant of ATCC 31532 allowing the detection of  
22 violacein in response to C6-HSL QS activation [24]. The *C. violaceum* CV026 QSI assay was  
23 conducted as previously described [23]. In brief, an overnight culture of the reporter strain  
24 was diluted in fresh sterile LB medium to an  $OD_{590nm} = 0.1$  and 100  $\mu$ l of this cell suspension  
25 was added to the wells of a 96-well MTP (Perkin Elmer). Kanamycin was added to a final

1 concentration of 20  $\mu\text{g/ml}$ . Activation of QS by AHL was tested by addition of C6-HSL (2.5  
2  $\mu\text{M}$ ) (Sigma). Sterile MilliQ water served as a negative control. To study inhibition using *C.*  
3 *violaceum* CV026, test compounds were added in selected sub-MIC concentrations. The MTP  
4 was then incubated for 48 h at 27°C to allow induction of violacein formation. The plates  
5 were then dried at 60°C until all medium had evaporated (6 h). The violacein was  
6 resolubilized by adding 100  $\mu\text{l}$  of DMSO to each well and the plates incubated on a lab shaker  
7 for two hours. The absorbance was measured at 590 nm using a Victor Wallac<sup>2</sup> multilabel  
8 counter (Perkin Elmer). *E. coli* JB523 contains the pJBA130 plasmid (expressing the green  
9 fluorescent protein GFP in response to C6-HSL) [1]. The *E. coli* JB523 QSI assay was carried  
10 out in a 96-well MTP. In brief, an overnight culture of the reporter strain was diluted in fresh  
11 sterile LB medium containing 4 % (w/v) NaCl to an  $\text{OD}_{590\text{nm}} = 0.1$  and 100  $\mu\text{l}$  of this cell  
12 suspension was added to the wells of a black 96-well MTP (Perkin Elmer). Tetracycline was  
13 added to a final concentration of 100  $\mu\text{g/ml}$ . Activation of QS by AHL was tested by addition  
14 of C6-HSL (5  $\mu\text{M}$ ) (Sigma). Sterile MilliQ water served as a negative control. To study  
15 inhibition using *E. coli* JB523, test compounds were added in selected sub-MIC  
16 concentrations. The MTP was then incubated for 24 h at 30°C and fluorescence from GFP  
17 expression was measured at  $\lambda_{\text{ex}}$  475 nm and  $\lambda_{\text{em}}$  515 nm using a Victor Wallac<sup>2</sup> multilabel  
18 counter (Perkin Elmer). The QSI selector strain *QSIS2*, previously developed by Rasmussen  
19 et al. (2005) is a *P. aeruginosa lasI rhII* double mutant harbouring pLasB-SacB1 encoding an  
20 AHL-induced killing system. A *QSIS2* QSI assay was developed in microtiter format based  
21 on the plate assay described by Rasmussen et al. (2005). An overnight culture of the reporter  
22 strain was diluted in fresh sterile ABT medium to an  $\text{OD}_{590\text{nm}} = 0.1$  and 50  $\mu\text{l}$  of this cell  
23 suspension was added to the wells of a 96-well MTP. Fifty  $\mu\text{l}$  LB supplemented with sucrose  
24 (56 mg/ml) was added to each well. Gentamycin was added in a concentration of 80  $\mu\text{g/ml}$ .  
25 Activation of QS by AHL's was tested by addition of 3-oxo-C12-HSL and C4-HSL (Sigma)

1 (200 nM each) to the wells. Sterile MilliQ water served as a negative control. To study  
2 inhibition using *QSYS2*, test compounds were added in selected concentrations. The MTP was  
3 then incubated for 10 h at 37°C and the absorbance was measured at 450 nm using a Victor  
4 Wallac<sup>2</sup> multilabel counter. The difference in growth of the negative control (without addition  
5 of compound and without AHL) and that of the positive control (without addition of  
6 compound and with AHL) was set at 100 %. Each compound was tested six times in each  
7 assay and each assay was repeated at least six times ( $n \geq 36$ ).

8

### 9 *2.5. Biofilm formation in 96-well MTP*

10 Biofilms were formed as previously described [4]. In brief, the Bcc strains were grown  
11 overnight in SCFM, centrifuged, resuspended in double concentrated SCFM (2xSCFM) and  
12 diluted to an  $OD_{590nm} = 0.1$  in 2xSCFM. Fifty  $\mu$ l of the diluted bacterial suspension was  
13 transferred to the wells of a round-bottomed 96-well microtiter plate (TPP). Negative controls  
14 received 50  $\mu$ l of CS. Positive controls received 50  $\mu$ l of the test compound. Bacteria were  
15 allowed to adhere and grow without agitation for 4 h at 37°C. After 4 h, plates were emptied  
16 and washed with sterile physiological saline (PS). After this washing step, negative control  
17 wells were filled with 50  $\mu$ L 2xSCFM and 50  $\mu$ l CS. Other wells were filled with 50  $\mu$ l  
18 2xSCFM and 50  $\mu$ l compound solution and the plate was incubated for 24 h at 37°C. The  
19 biomass was quantified by crystal violet (CV) staining, as described previously [29]. In brief,  
20 plates were rinsed with sterile PS, biofilms were fixed by adding 100  $\mu$ l 99 % methanol for 15  
21 min, after which the methanol was removed and plates were air-dried. Biofilms were then  
22 stained with 100  $\mu$ l CV (Pro-lab Diagnostics, Richmond Hill, ON, Canada). After 20 min, CV  
23 was removed and wells were filled with 150  $\mu$ l 33 % acetic acid (Sigma-Aldrich). The  
24 absorbance was measured at 590 nm using a Wallac Victor<sup>2</sup> multilabel counter.  
25 Quantification of the number of metabolically active (i.e. living) cells in the biofilm was done



1 using resazurin assay (CellTiter-Blue CTB) [29]. In brief, wells were rinsed after 24 h biofilm  
2 formation and 100  $\mu$ l PS was added, followed by addition of 20  $\mu$ l CellTiter-Blue (CTB)  
3 (Promega, Leiden, The Netherlands) solution. After 60 min, fluorescent (excitation and  
4 emission filters of 486 nm and 535 nm) was measured using a Wallac Victor<sup>2</sup> multilabel  
5 counter. Each compound was tested six times in each assay and each assay was repeated at  
6 least four times ( $n \geq 24$ ).

7

## 8 *2.6. Biofilm formation on silicone disks and evaluation of the effect on biofilms*

9 For the quantification of the number of adhered cells and number of detached cells, *B.*  
10 *cenocepacia* LMG16656 was allowed to form biofilms on silicone disks (Q7-4735; Dow  
11 Corning) in a 24-well plate in the presence and absence of test compounds. In brief, 500  $\mu$ L of  
12 the diluted bacterial suspension was transferred to the wells of a 24-well plate (TPP,  
13 Trasadingen, Switzerland) containing a sterile silicone disk. Subsequently, 500  $\mu$ l of the test  
14 compound was added. To control wells we added 500  $\mu$ l CS. Bacteria were allowed to adhere  
15 and grow without agitation for 4 h at 37°C. After 4 h, the growth medium was removed, the  
16 disks were rinsed with 0.9 % (w/v) NaCl and 500  $\mu$ l 2xSCFM and 500  $\mu$ l test compound was  
17 added. The plate was further incubated for 24 h at 37°C. The number of culturable sessile  
18 cells and the total number of cells present on the silicone disks were determined after 4 h and  
19 24 h by plating as previously described [7]. In brief, each disk was transferred to 10 ml 0.9 %  
20 (w/v) NaCl. The tubes were subjected three times to 30 s of sonication (Branson 3510,  
21 42 kHz, 100 W; Branson Ultrasonics Corp., Danbury, CT) and 30 s of vortex mixing to  
22 remove the biofilm cells from the disks. Using this procedure, all cells are removed from the  
23 disks and clumps of cells were broken apart (data not shown). The number of sessile  
24 *Burkholderia cenocepacia* LMG16656 cells was quantified by plating on TSA. All plates  
25 were incubated at 37°C for 24 h and the number of CFU per disk was calculated by counting

1 colonies on the plates. To quantify the number of bacteria released from the biofilms on the  
2 silicone disks and the number of cells adhering to the disk, the number of cells in the medium  
3 and the rinsing solution after 4 and 24 h was determined by plating. Each compound was  
4 tested on three disks in each assay and each assay was repeated at least three times ( $n \geq 9$ ).

5

### 6 *2.7. Confocal laser scanning microscopy (CLSM)*

7 Biofilms of *Burkholderia cenocepacia* LMG16656 were formed on silicone disks as  
8 described above. The effect of “compound 3” on biofilm formation was evaluated after 4 h of  
9 adhesion and after 24 h of biofilm formation. For CLSM, the biofilm-covered disks were  
10 placed in the wells of a 24-well plate (Greiner bio-one, Longwood, FL, USA) and covered  
11 with 1 ml of PS containing 3  $\mu$ l of SYTO9. The plates were incubated for 15 min at room  
12 temperature and the biofilm was visualised with a Nikon C1 confocal laser scanning module  
13 attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux, Brussels,  
14 Belgium) equipped with a Plan Apo VC 60x 1.4 NA oil immersion objective and suitable  
15 optical elements to obtain fluorescent and differential interference contrast (DIC) transmission  
16 images. SYTO9 was excited with the Argon ion 488 nm laser line and emission light was  
17 collected using a 500-530 nm band pass filter and Z-stacks were recorded. Tests were  
18 performed on at least two disks for each situation and representative images are shown.

19

### 20 *2.8. Statistics*

21 The normal distribution of the data was checked using the Shapiro-Wilk test. Normally  
22 distributed data were analyzed using an Independent samples T-tests. Non-normally  
23 distributed data were analyzed using the Mann-Whitney U test. Statistics were performed  
24 using the SPSS software, version 15.0 (SPSS, Chicago, IL, USA).

25

### 1 3. Results

#### 2 3.1. AHL-QSI

3 The MICs for all compounds were determined (data not shown). In all further experiments,  
4 the compounds were used in concentrations that were below this MIC. In order to confirm  
5 QSI, we retested the 22 QS inhibitors in the three QSI detection systems (Table 1). Maximum  
6 levels of inhibition in the three assays were found for the following compounds: esculetin (55  
7  $\pm 23$  %) (*C. violaceum* CV026 QSI assay), esculetin (50  $\pm 20$  %), baicalin hydrate (50  $\pm 7$  %)  
8 and p-benzoquinon (41  $\pm 17$  %) (*E. coli* JB523 QSI assay), esculetin (67  $\pm 2$  %), “compound  
9 3” (58  $\pm 4$  %), “compound 1” (38  $\pm 17$  %) and esculin hydrate (29  $\pm 8$  %) (*P. aeruginosa*  
10 QSI assay).

11

#### 12 3.2. Quantification of the effect of QSI on biofilm formation in 96-well MTPs

13 There was a significant decrease in biofilm formation relative to the control when the  
14 different strains were grown in the presence of various QS inhibitory compounds (Table 2).  
15 The use of “compound 3” (500  $\mu$ M), baicalin hydrate (500  $\mu$ M) and farnesol (2500  $\mu$ M)  
16 resulted in a reduction of biofilm biomass in all strains tested, as quantified by CV staining.  
17 The biofilm biomass in any of the four strains was not reduced by 2-amino-6-chlorobenzoic  
18 acid, 2-amino-4-chlorobenzoic acid and (+)- catechin. The 16 other compounds affected the  
19 biofilm biomass in at least one of the strains tested. Using CTB-staining, nine out of the 22  
20 compounds were found to reduce the number of metabolically active biofilm cells in all four  
21 strains tested. In addition, all 22 compounds significantly affected the number of  
22 metabolically active cells in at least one strain. In contrast to what was observed for the other  
23 compounds, the use of tobramycin (2  $\mu$ M) resulted in a moderate but significant increase in  
24 biomass and numbers of metabolically active cells in *B. multivorans* LMG17588 and *B.*  
25 *cenocepacia* LMG18828.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25

### 3.3. Effect of QS inhibitors on sessile cell numbers and detachment

After 4 h, the number of cells present on the disks and in the growth medium did not significantly differ between the treated and untreated disks (Table 3). However, significantly more cells were removed during the rinsing step after 4 h from the disks treated with azithromycin, cinnamaldehyde, farnesol and indole. After 24 h, significantly fewer cells were present on the silicone disks treated with QS inhibitory compounds compared to the control (Table 3). In contrast, after 24 h a significantly higher number of colony forming units (CFU) was present in the growth medium of the biofilms treated with azithromycin, ceftazidime, “compound 3”, farnesol and indole compared to the growth medium of the control biofilm (Table 3). The number of cells removed during the final rinsing step was significantly higher for the biofilms treated with azithromycin, cinnamaldehyde and farnesol in comparison to the control (Table 3). Total numbers of CFU (both sessile and planktonic) were not significantly different after 4 h and 24 h of growth.

### 3.4. Confocal scanning laser microscopy

Consistent with the results obtained by plating, no differences between the treated biofilm and the untreated control were observed after 4 h of adhesion (Fig 1A). However, after 24 h CLSM revealed clear differences in *B. cenocepacia* LMG16656 biofilm structure between biofilms treated with “compound 3” (500  $\mu$ M) and the untreated control (Fig 1B), as the former biofilm was less structured and contained less cells.

## 1 **4. Discussion**

2

### 3 *4.1. AHL-QSI*

4 Since the compounds used in this study were previously reported to affect QS in at least one  
5 QS reporter, we expected that they would exhibit an effect in at least one of the QSI detection  
6 systems used in this study. However, we often observed only a moderate QS-inhibitory effect.  
7 This may be due to the lower concentrations used in the present study in order to perform  
8 experiments with sub-MIC concentrations for all test strains. A second possible explanation  
9 concerns the mechanism by which the compounds affect the QS system. For example,  
10 azithromycin reportedly affects several QS regulated phenotypes in *P. aeruginosa* [12; 40] by  
11 decreasing LasI and RhII gene expression. However, since the biosensor strains used in the  
12 present study are unable to produce signal molecules themselves, the latter were externally  
13 added so that no effect at the level of the biosynthesis of signal molecules can be observed.  
14 Recently, Skindersoe et al. (2008) found that several antibiotics, including azithromycin and  
15 ceftazidime influenced QS in *P. aeruginosa* by changing membrane permeability, thereby  
16 influencing the uptake of signal molecules. However, this effect could not be confirmed in the  
17 present study. A third explanation for the inability of some compounds to interfere with QS in  
18 these detection systems could lie in the specificity of the detection system and/or of the  
19 compound. For example, cinnamaldehyde seems to have no effect on *P. aeruginosa* QS, using  
20 the *QSIS2* detection system, while moderate effects were observed in the other two QS  
21 detection systems. Niu et al. (2006) previously showed that cinnamaldehyde interfered with  
22 the binding of short chain signal molecules (like C6-HSL used as a signal molecule in *E. coli*  
23 JB523 and *C. violaceum* CV026 detection system), but that it failed to substantially reduce the  
24 binding of longer chain signal molecules like 3-oxo-C12-HSL. In addition, farnesol and  
25 various halogenated benzoic acids were previously found to influence PQS related QS

1 systems in *P. aeruginosa*, but the systems used here only detect a direct effect on the  
2 interaction between AHL signal molecules and the AHL-receptor. However, it should be  
3 noted that 2-amino-6-chlorobenzoic acid affect QS in the *E. coli* JB523 QSI detection system  
4 and that 2-amino-6-fluorobenzoic acid inhibited QS in both the *E. coli* JB523 and *C.*  
5 *violaceum* CV026 QSI systems.

6

#### 7 4.2. Effect of QS inhibitors on biofilms

8 Results from several reports suggest that targeting QS could be a new strategy to fight biofilm  
9 infections [25]. However, only few QS inhibitory compounds have been evaluated in sub-  
10 MIC concentrations for their anti-biofilm effect against Bcc biofilms. Several QS inhibiting  
11 polyphenols and “compound 3” influenced biofilm formation of *B. cenocepacia* H111 [15;  
12 32]. For the other QS inhibitory compounds the effect on biofilms has not been evaluated at  
13 all or only on non-Bcc species [12; 17; 34; 39; 44]. For this reason we wanted to explore  
14 whether QS inhibitory compounds (when used in sub-MIC concentrations) could affect Bcc  
15 biofilm formation in this study. To this end we selected 22 established QS inhibitory  
16 compounds and evaluated their antibiofilm effect against the two Bcc species most often  
17 involved in CF infections. As can be seen in Table 2, 21 out of 22 components tested resulted  
18 in a reduction in biofilm formation in at least one strain. Interestingly, several compounds  
19 were shown to affect biofilm formation in all the strains tested, while others did so in one or a  
20 few strains only. Most of the compounds reduced the total biofilm biomass (as quantified by  
21 CV staining), as well as the number of metabolically active cells (as quantified by CTB  
22 staining). However, some compounds that yielded in a reduction of the number of living cells  
23 failed to reduce the total biomass (e.g. (+)-catechin). The use of tobramycin (2  $\mu$ M) resulted  
24 in an increase in biofilm biomass and in the number of metabolically active cells in two  
25 strains, i.e. *B. multivorans* LMG17588 and *B. cenocepacia* LMG18828. This increase was

1 previously also observed for *P. aeruginosa* [11]. Since all compounds were tested in sub-MIC  
2 concentrations, the reduction in metabolically active cells can not be explained by lethal  
3 effects. Indeed, when using SYTO9 (a DNA-binding dye that stains both living and dead  
4 cells) a reduction in fluorescence was observed in treated biofilms. As the fluorescence of  
5 dead cells is approximately equal to that of living cells, this reduction suggests that treatment  
6 with QS inhibitors results in lower cell numbers through reduced attachment and/or increased  
7 detachment. This was investigated by determining the number of CFU present on the disks  
8 (after 4 and 24 h of growth), by determining the number of CFU present in the surrounding  
9 growth medium and in the rinsing solution (after 4 h and 24 h) and by visualizing the biofilms  
10 with CSLM (after 4 and 24 h of growth). Significantly lower amounts of cells were indeed  
11 present on the disks treated with the compound after 24 h, while no differences were observed  
12 after 4 h. In addition, more detached cells were found in the growth medium and rinsing  
13 solution after 24 h. These data suggest that the QS inhibitors do not exert their effect during  
14 initial stages of attachment, but rather promote detachment at later stages. This is in  
15 agreement with previously published data. Huber et al. (2001) found no difference in initial  
16 attachment for a *B. cenocepacia* H111 CepIR mutant compared to the wild type but suggested  
17 that a functional QS system was required for maturation of the biofilm. In addition,  
18 biosurfactant production, which was previously found to be regulated by QS in several  
19 bacteria [19; 27] possibly influences detachment from surfaces [33]. However, whether these  
20 QS inhibitory compounds indeed exert their effect through a change in biosurfactant  
21 production in Bcc biofilms remains to be determined.

22 Whether these compounds, either alone or in combination with conventional antimicrobial  
23 agents, will ever be useful as anti-biofilm agents remains to be determined in future studies.  
24 However, there are indications that there may be a role for these compounds, even though  
25 their anti-biofilm effect is only moderate. For example, Tomlin et al. [41] showed that, despite

1 the fact that the changes observed with their biofilm model were not biologically relevant,  
2 disrupting the QS system made sessile *B. cenocepacia* cells more susceptible to antibiotics.  
3 This supports the idea that a decrease in the overall structural stability of the biofilm, although  
4 it may not be biologically relevant at first sight, may allow for more efficacious removal of  
5 bacteria by other means.

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25



## 1 **5. Acknowledgements**

2 The authors like to thank Dr. M. Givskov for kindly providing the *P. aeruginosa* QSIS2  
3 strain, D. Vercauteren for the assistance with the CLSM and K. Van Bocxlaer and B.  
4 Beernaert for excellent technical assistance. This work was supported by the Institute for the  
5 Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) and  
6 FWO-Vlaanderen.

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

## 1 6. References

- 2 1. Andersen, J.B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B.B.,  
3 Molin, S. and Givskov, M. (2001) *gfp*-based *N*-acyl homoserine lactone sensor systems for  
4 detection of bacterial communication. *Appl. Env. Microbiol.* 67,575-585.  
5
- 6 2. Baldwin, A., Sokol, P.A., Parkhill, J., Mahenthiralingam, E. (2004) The *Burkholderia*  
7 *cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and  
8 metabolism-associated genes in *Burkholderia cenocepacia*. *Infect. Immun.* 72(3), 1537-1547.  
9
- 10 3. Bassler, B.L. (1999) How bacteria talk to each other: regulation of gene expression by  
11 quorum sensing. *Curr. Opin. Microbiol.* 2(6), 582-7.  
12
- 13 4. Brackman, G., Defoirdt, T., Miyamoto, C., Van Calenbergh, S., Bossier, P., Nelis, H.J.,  
14 Coenye, T. (2008) Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in  
15 *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator  
16 LuxR. *BMC Microbiol.* 8(1):149.  
17
- 18 5. Caraher, E., Reynolds, G., Murphy, P., McClean, S., Callaghan, M. (2007) Comparison of  
19 antibiotic susceptibility of *Burkholderia cepacia* complex organisms when grown  
20 planktonically or as biofilm *in vitro*. *Eur. J. Clin. Microbiol. Infect. Dis.* 26(3), 213-216.  
21
- 22 6. Coenye, T., Vandamme, P., Govan, J.R., LiPuma, J.J. (2001) Taxonomy and identification  
23 of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* 39(10),3427-36.  
24

- 1 7. Coenye, T., De Prijck, K., De Wever, B., Nelis, H.J. (2008) Use of the modified Robbins  
2 device to study the *in vitro* biofilm removal efficacy of NitrAdine, a novel disinfecting  
3 formula for the maintenance of oral medical devices. *J. Appl. Microbiol.* 105(3), 733-40.  
4
- 5 8. Conway, B.A., Venu, V., Speert, D.P. (2002) Biofilm formation and acyl homoserine  
6 lactone production in the *Burkholderia cepacia* complex. *J. Bacteriol.* 184(20), 5678-5685.  
7
- 8 9. Cugini, C., Calfee, M.W., Farrow, J.M. 3rd, Morales, D.K., Pesci, E.C., Hogan, D.A.  
9 (2007) Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas*  
10 *aeruginosa*. *Mol. Microbiol.* 65(4), 896-906.  
11
- 12 10. Garske, L.A., Beatson, S.A., Leech, A.J., Walsh, S.L., Bell, S.C. (2004) Sub-inhibitory  
13 concentrations of ceftazidime and tobramycin reduce the quorum sensing signals of  
14 *Pseudomonas aeruginosa*. *Pathology.* 36(6), 571-5.  
15
- 16 11. Hoffman, L.R., D'Argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A., Miller, S.I.  
17 (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature.* 436(7054),  
18 1171-5.  
19
- 20 12. Hoffmann, N., Lee, B., Hentzer, M., Rasmussen, T.B., Song, Z., Johansen, H.K., Givskov,  
21 M., Hoiby, N. (2007) Azithromycin blocks quorum sensing and alginate polymer formation  
22 and increases the sensitivity to serum and stationary-growth-phase killing of *Pseudomonas*  
23 *aeruginosa* and attenuates chronic *P. aeruginosa* lung infection in *Cftr(-/-)* mice. *Antimicrob.*  
24 *Agents Chemother.* 51(10), 3677-87.  
25

- 1 13. Honraet, K., Nelis, H.J. (2006) Use of the modified robbins device and fluorescent  
2 staining to screen plant extracts for the inhibition of *S. mutans* biofilm formation. J.  
3 Microbiol. Methods. 64(2), 217-224.  
4
- 5 14. Huber, B., Riedel, K., Hentzer, M., Heydorn, A., Gotschlich, A., Givskov, M., Molin, S.,  
6 Eberl, L. (2001). The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls  
7 biofilm formation and swarming motility. Microbiology. 147, 2517-28.  
8
- 9 15. Huber, B., Eberl, L., Feucht, W., Polster, J. (2003). Influence of polyphenols on bacterial  
10 biofilm formation and quorum-sensing. Z Naturforsch [C]. 58(11-12), 879-84.  
11
- 12 16. Keshavan, N.D., Chowdhary, P.K., Haines, D.C., Gonzalez, J.E. (2005). L-canavanine  
13 made by *Medicago sativa* interferes with quorum sensing in *Sinorhizobium meliloti*. J.  
14 Bacteriol. 187(24), 8427-36.  
15
- 16 17. Koo, H., Hayacibara, M.F., Schobel, B.D., Cury, J.A., Rosalen, P.L., Park, Y.K., Vacca-  
17 Smith, A.M., Bowen, W.H. (2003) Inhibition of *Streptococcus mutans* biofilm accumulation  
18 and polysaccharide production by apigenin and tt-farnesol. J. Antimicrob. Chemother. 52(5),  
19 782-9.  
20
- 21 18. Lesic, B., Lepine, F., Deziel, E., Zhang, J., Zhang, Q., Padfield, K., Castonguay, M.H.,  
22 Milot, S., Stachel, S., Tzika, A.A., Tompkins, R.G., Rahme, L.G. (2007) Inhibitors of  
23 pathogen intercellular signals as selective anti-infective compounds. PLoS Pathog. 3(9),  
24 1229-39.  
25

- 1 19. Lindum, P.W., Anthoni, U., Christophersen, C., Eberl, L., Molin, S., Givskov, M. (1998)  
2 N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide  
3 biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. J. Bacteriol.  
4 180(23), 6384-8.
- 5
- 6 20. LiPuma, J.J., Spilker, T., Gill, L.H., Campbel, P.W. 3<sup>rd</sup>, Liu, L., Mahenthalingam, E.  
7 (2001) Disproportionate distribution of *Burkholderia cepacia* complex species and  
8 transmissibility markers in cystic fibrosis. Am. J. Respir. Crit. Care Med. 164(1), 92-6.
- 9
- 10 21. Mahenthalingam, E., Vandamme, P. (2005) Taxonomy and pathogenesis of the  
11 *Burkholderia cepacia* complex. Chron. Respir. Dis. 2(4), 209-17.
- 12
- 13 22. Malott, R.J., Baldwin, A., Mahenthalingam, E., Sokol, P.A. (2005) Characterization of  
14 the *cciIR* quorum-sensing system in *Burkholderia cenocepacia*. Infect. Immun. 73(8), 4982-  
15 92.
- 16
- 17 23. Martinelli, D., Grossmann, G., Séquin, U., Brandl, H. and Bachofen, R. (2004). Effects of  
18 natural and chemically synthesised furanones on quorum sensing in *Chromobacterium*  
19 *violaceum*. BMC Microbiol 4:25.
- 20
- 21 24. McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin,  
22 M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S.A.B. and Williams, P. (1997) Quorum  
23 sensing and *Chromobacterium violaceum* : exploitation of violacein production and inhibition  
24 for the detection of *N*-acylhomoserine lactones. Microbiology 143, 3703-3711.

25

- 1 25. Musk, D.J. Jr, Hergenrother, P.J. (2006) Chemical countermeasures for the control of  
2 bacterial biofilms: effective copounds and promising targets. *Curr. Med. Chem.* 13(18), 2163-  
3 77.  
4
- 5 26. Niu, C., Alfre, S., Gilbert, E.S. (2006) Subinhibitory concentrations of cinnamaldehyde  
6 interfere with quorum sensing. *Lett. Appl. Microbiol.* 43, 489-494  
7
- 8 27. Ochsner, U.A., Reiser, J. (1995) Autoinducer-mediated regulation of rhamnolipid  
9 biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A.* 92(14), 6424-  
10 8.  
11
- 12 28. Palmer, K.L., Aye, L.M., Whiteley, M. (2007) Nutritional cues control *Pseudomonas*  
13 *aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol.* 189(22), 8079-87.  
14
- 15 29. Peeters, E., Nelis, H.J., Coenye, T. (2007) Comparison of multiple methods for  
16 quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods* 72,  
17 157-165.  
18
- 19 30. Pesci, E.C., Milbank, J.B.J., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P.,  
20 Iglewski, B.H. (1999) Quinolone signalling in the cell-to-cell communication system of  
21 *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 96,11229-11234.  
22
- 23 31. Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristofferson, P., Kôte, M.,  
24 Nielsen, J., Eberl, L., Givskov, M. (2005) Screening for quorum-sensing inhibitors (QSI) by use  
25 of a novel genetic system, the QSI selector. *J. Bacteriol.* 187,1799-1814.

- 1
- 2 32. Riedel, K., Köthe, M., Kramer, B., Saeb, W., Gotschlich, A., Ammendola, A., Eberl, L.
- 3 (2006) Computer-aided design of agents that inhibit the *cep* quorum-sensing system of
- 4 *Burkholderia cenocepacia*. *Antimicrob. Agents Chemother.* 50(1),318-23.
- 5
- 6 33. Rosenberg, E., Ron, E.Z. (1999) High- and low-molecular-mass microbial surfactants.
- 7 *Appl. Microbiol. Biotechnol.* 52(2), 154-62.
- 8
- 9 34. Rudrappa, T., Bais, H.P. (2008) Curcumin, a known phenolic from *Curcuma longa*,
- 10 attenuates the virulence of *Pseudomonas aeruginosa* PAO1 in whole plant and animal
- 11 pathogenicity Models. *J. Agric. Food Chem.* 56(6), 1955-62.
- 12
- 13 35. Savoia, D., Zucca, M. (2007) Clinical and environmental *Burkholderia* strains: biofilm
- 14 production and intracellular survival. *Curr. Microbiol.* 54(6), 440-444.
- 15
- 16 36. Skindersoe, M.E., Alhede, M., Phipps, R., Yang, L., Jensen, P.O., Rasmussen, T.B.,
- 17 Bjarnsholt, T., Tolker-Nielsen, T., Hoiby, N., Givskov, M. (2008) Effects of antibiotics on
- 18 quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 52(10), 3648-
- 19 63.
- 20
- 21 37. Sokol, P.A., Malott, R.J., Riedel, K, Eberl, L. (2007) Communication systems in the
- 22 genus *Burkholderia*: global regulators and targets for novel antipathogenic drugs. *Future*
- 23 *Microbiol.* 2(5), 555-563.
- 24

- 1 38. Speert, D.P., Henry, D., Vandamme, P., Corey, M., Mahenthiralingam, E. (2002)  
2 Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada.  
3 Emerg. Infect. Dis. 8, 181–7.  
4
- 5 39. Starner, T.D., Shrout, J.D., Parsek, M.R., Appelbaum, P.C., Kim, G. (2008) Subinhibitory  
6 concentrations of azithromycin decrease nontypeable *Haemophilus influenzae* biofilm  
7 formation and diminish established biofilms. Antimicrob. Agents Chemother. 52(1), 137-45.  
8
- 9 40. Tateda, K., Cote, R., Pechere, J.C., Kohler, T., Yamaguchi, K., Van Delden, C. (2001)  
10 Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. Antimicrob. Agents  
11 Chemother. (6), 1930-3.  
12
- 13 41. Tomlin, K.L., Malott, R.J., Ramage, G., Storey, D.G., Sokol, P.A., Ceri, H (2005)  
14 Quorum-sensing mutations affect attachment and stability of *Burkholderia cenocepacia*  
15 biofilms. Appl. Environ. Microbiol. 71(9), 5208-18.  
16
- 17 42. Vial, L., Lepine, F., Milot, S., Groleau, M.C., Dekimpe, V., Woods, D.E., Deziel, E.  
18 (2008) *Burkholderia pseudomallei*, *B. thailandensis* and *B. ambifaria* produce 4-hydroxy-2-  
19 alkylquinoline (HAQ) analogues with a methyl group at the 3 position that is required for  
20 quorum sensing regulation. J. Bacteriol. 190(15), 5339-52.  
21
- 22 43. Wang, W.B., Lai, H.C., Hsueh, P.R., Chiou, R.Y., Lin, S.B., Liaw, S.J. (2006) Inhibition  
23 of swarming and virulence factor expression in *Proteus mirabilis* by resveratrol. J. Med.  
24 Microbiol. 55, 1313-21.  
25



- 1 44. Zeng, Z., Qian, L., Cao, L., Tan, H., Huang, Y., Xue, X., Shen, Y., Zhou, S. (2008)  
2 Virtual screening for novel quorum sensing inhibitors to eradicate biofilm formation of  
3 *Pseudomonas aeruginosa*. Appl. Microbiol. Biotechnol. 79(1), 119-26.  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24

1 Figure 1: Representative confocal images of *B. cenocepacia* LMG16656 biofilms treated with  
2 “compound 3” (500  $\mu$ M) or an untreated control. Biofilms were stained using SYTO9 and  
3 CLSM pictures were taken after 4 h adhesion (A) and after 24 h of biofilm growth (B) (at  
4 both time points following the rinsing step). The larger central plots are fluorescence  
5 projections. Shown in the right and lower frames are vertical sections through the biofilms.  
6 The scale bar represents 10  $\mu$ m.

- 1 Table 1 : QS inhibitory effects of several QS inhibitors in the three QSI detection systems. QSI is expressed as % inhibition ( $\pm$  standard deviation).
- 2 NS: not significant ( $p > 0.05$ ; Independent Sample T-test).

Compound	Conc ( $\mu$ M)	% inhibition ( $\pm$ SD)		
		<i>C. violaceum</i> CV026	<i>E. coli</i> JB523	<i>P. aeruginosa</i> QSI2
4CABA	50	NS	NS	NS
6CABA	50	NS	22 $\pm$ 23	NS
6FABA	50	11 $\pm$ 11	32 $\pm$ 22	NS
Azithromycin	2	NS	22 $\pm$ 12	NS
Baicalein	1	15 $\pm$ 12	31 $\pm$ 28	NS
Baicalin hydrate	100	25 $\pm$ 2	50 $\pm$ 7	7 $\pm$ 11
p-Benzoquinon	20	11 $\pm$ 12	41 $\pm$ 17	15 $\pm$ 8
L-canavanine	62.5	14 $\pm$ 4	9 $\pm$ 3	NS
(+)- Catechin	312	11 $\pm$ 3	8 $\pm$ 3	NS
Ceftazidime hydrate	0.075	9 $\pm$ 5	11 $\pm$ 15	NS
Cinnamaldehyde	100	16 $\pm$ 15	15 $\pm$ 7	NS
“Compound 1”	500	29 $\pm$ 13	22 $\pm$ 18	38 $\pm$ 17
“Compound 3”	500	9 $\pm$ 4	NS	58 $\pm$ 4
Curcumin	500	18 $\pm$ 12	37 $\pm$ 14	NS
(-)- Epigallocatechin gallate	50	16 $\pm$ 12	26 $\pm$ 4	NS
Esculetin	500	55 $\pm$ 23	50 $\pm$ 20	67 $\pm$ 2
Esculin hydrate	500	25 $\pm$ 13	19 $\pm$ 13	29 $\pm$ 8
Farnesol	500	NS	NS	NS
Indole	312	13 $\pm$ 4	19 $\pm$ 14	14 $\pm$ 14
4-nitropyridine oxide	10	NS	NS	21 $\pm$ 4
Resveratrol	25	14 $\pm$ 3	19 $\pm$ 14	NS
Tobramycin	0.5	14 $\pm$ 1	16 $\pm$ 4	NS

- 1 Table 2: The effect of QSI on biofilm biomass (CV) and amount of metabolically active cells (CTB) after 24 h of biofilm formation. The effect is
- 2 expressed as % inhibition ( $\pm$  SD). NS: not significant ( $p > 0.05$ ; Independent Sample T-test).

Compound (conc)	percentage inhibition CV signal ( $\pm$ SD)				percentage inhibition CTB signal ( $\pm$ SD)			
	<i>B. multivorans</i>		<i>B. cenocepacia</i>		<i>B. multivorans</i>		<i>B. cenocepacia</i>	
	LMG13010	LMG17588	LMG18828	LMG16656	LMG13010	LMG17588	LMG18828	LMG16656
<b>4CABA (50 <math>\mu</math>M)</b>	NS	NS	NS	NS	NS	NS	NS	25 $\pm$ 2
<b>6CABA (50 <math>\mu</math>M)</b>	NS	NS	NS	NS	NS	NS	NS	19 $\pm$ 9
<b>6FABA (50 <math>\mu</math>M)</b>	NS	25 $\pm$ 8	NS	NS	NS	28 $\pm$ 13	NS	29 $\pm$ 4
<b>Azithromycin (2 <math>\mu</math>M)</b>	NS	NS	NS	15 $\pm$ 16	25 $\pm$ 10	55 $\pm$ 3	32 $\pm$ 5	40 $\pm$ 11
<b>Baicalein (1 <math>\mu</math>M)</b>	NS	13 $\pm$ 7	NS	NS	NS	28 $\pm$ 8	NS	16 $\pm$ 3
<b>Bacalin hydrate (100 <math>\mu</math>M)</b>	48 $\pm$ 7	37 $\pm$ 9	62 $\pm$ 2	11 $\pm$ 19	46 $\pm$ 5	28 $\pm$ 12	54 $\pm$ 8	16 $\pm$ 4
<b>p-benzoquinon (100 <math>\mu</math>M)</b>	NS	NS	21 $\pm$ 8	31 $\pm$ 36	NS	NS	18 $\pm$ 6	18 $\pm$ 24
<b>L-Canavanine (20 <math>\mu</math>M)</b>	NS	19 $\pm$ 7	14 $\pm$ 8	43 $\pm$ 25	11 $\pm$ 13	29 $\pm$ 9	10 $\pm$ 4	26 $\pm$ 20
<b>(+)- Catechin (1000 <math>\mu</math>M)</b>	NS	NS	NS	NS	26 $\pm$ 14	35 $\pm$ 11	30 $\pm$ 6	48 $\pm$ 17
<b>Ceftazidime hydrate (1 <math>\mu</math>M)</b>	8 $\pm$ 12	29 $\pm$ 5	NS	41 $\pm$ 21	28 $\pm$ 11	54 $\pm$ 12	37 $\pm$ 9	22 $\pm$ 15
<b>Cinnamaldehyde (250 <math>\mu</math>M)</b>	NS	11 $\pm$ 10	12 $\pm$ 15	31 $\pm$ 29	NS	11 $\pm$ 12	22 $\pm$ 4	12 $\pm$ 16
<b>“Compound 1” (500<math>\mu</math>M)</b>	17 $\pm$ 24	39 $\pm$ 9	NS	53 $\pm$ 18	20 $\pm$ 9	22 $\pm$ 8	NS	25 $\pm$ 14
<b>“Compound 3” (500 <math>\mu</math>M)</b>	37 $\pm$ 10	26 $\pm$ 14	19 $\pm$ 10	54 $\pm$ 8	22 $\pm$ 9	35 $\pm$ 10	NS	45 $\pm$ 9
<b>Curcumin (500 <math>\mu</math>M)</b>	23 $\pm$ 12	24 $\pm$ 6	NS	NS	21 $\pm$ 10	14 $\pm$ 4	NS	NS
<b>(-)- Epigallocatechin gallate (0.4 <math>\mu</math>M)</b>	NS	16 $\pm$ 6	24 $\pm$ 9	10 $\pm$ 14	NS	40 $\pm$ 7	20 $\pm$ 15	26 $\pm$ 8
<b>Esculetin (500 <math>\mu</math>M)</b>	25 $\pm$ 10	26 $\pm$ 13	NS	10 $\pm$ 15	19 $\pm$ 15	11 $\pm$ 6	11 $\pm$ 6	10 $\pm$ 5
<b>Esculin hydrate (500 <math>\mu</math>M)</b>	22 $\pm$ 13	46 $\pm$ 4	NS	9 $\pm$ 13	17 $\pm$ 10	9 $\pm$ 9	NS	NS
<b>Farnesol (2500 <math>\mu</math>M)</b>	42 $\pm$ 10	38 $\pm$ 5	22 $\pm$ 10	50 $\pm$ 13	26 $\pm$ 8	41 $\pm$ 14	41 $\pm$ 3	58 $\pm$ 11
<b>Indole (312 <math>\mu</math>M)</b>	NS	NS	NS	23 $\pm$ 22	12 $\pm$ 8	19 $\pm$ 13	28 $\pm$ 5	19 $\pm$ 11
<b>4-nitropyridine N-oxide (8 <math>\mu</math>M)</b>	NS	14 $\pm$ 6	NS	26 $\pm$ 22	NS	27 $\pm$ 6	NS	21 $\pm$ 3
<b>Resveratrol (25 <math>\mu</math>M)</b>	NS	12 $\pm$ 8	9 $\pm$ 13	38 $\pm$ 18	17 $\pm$ 17	25 $\pm$ 11	25 $\pm$ 3	30 $\pm$ 16
<b>Tobramycin (2 <math>\mu</math>M)</b>	NS	-16 $\pm$ 9	-15 $\pm$ 11	NS	NS	-12 $\pm$ 18	-9 $\pm$ 6	NS

- 1 Table 3: Numbers of cells present after 4 h and 24 h in the growth medium (M), rinsing solution (RS) and on the disks as well as total cell  
 2 numbers (TCN) present in the wells. \*:  $p < 0.05$  (Mann-Whitney U)

Numbers of cells present after 4 h				
Compound	M (CFU/ml) ( $\times 10^8$ )( $\pm$ SD)	RS (CFU/ml) ( $\times 10^8$ ) ( $\pm$ SD)	Disks (CFU/ Disk) ( $\times 10^8$ )( $\pm$ SD)	TCN (CFU/well) ( $\times 10^8$ )( $\pm$ SD)
CTRL	1.01 $\pm$ 0.17	0.48 $\pm$ 0.10	1.34 $\pm$ 0.37	2.83 $\pm$ 0.42
Azithromycin (2 $\mu$ M)	0.89 $\pm$ 0.28	0.71 $\pm$ 0.21*	1.15 $\pm$ 0.37	2.76 $\pm$ 0.44
Ceftazidime hydrate (1 $\mu$ M)	0.99 $\pm$ 0.48	0.54 $\pm$ 0.23	1.28 $\pm$ 0.62	2.81 $\pm$ 0.78
Cinnamaldehyde (250 $\mu$ M)	1.04 $\pm$ 0.05	0.74 $\pm$ 0.13*	1.12 $\pm$ 0.37	2.97 $\pm$ 0.41
“Compound 3” (500 $\mu$ M)	1.00 $\pm$ 0.04	0.52 $\pm$ 0.13	1.14 $\pm$ 0.14	2.65 $\pm$ 0.24
Farnesol (2500 $\mu$ M)	0.94 $\pm$ 0.06	0.80 $\pm$ 0.27*	1.15 $\pm$ 0.45	3.18 $\pm$ 0.53
Indole (312 $\mu$ M)	0.82 $\pm$ 0.49	0.71 $\pm$ 0.13*	1.34 $\pm$ 0.11	2.96 $\pm$ 0.60
Numbers of cells present after 24 h				
Compound	M (CFU/ml) ( $\times 10^8$ )( $\pm$ SD)	RS (CFU/ml) ( $\times 10^8$ )( $\pm$ SD)	Disks (CFU/ Disk) ( $\times 10^8$ )( $\pm$ SD)	TCN (CFU/well) ( $\times 10^8$ )( $\pm$ SD)
CTRL	1.36 $\pm$ 0.12	0.92 $\pm$ 0.24	1.99 $\pm$ 0.29	4.27 $\pm$ 0.40
Azithromycin (2 $\mu$ M)	2.33 $\pm$ 0.33*	1.63 $\pm$ 0.55*	0.71 $\pm$ 0.12*	4.68 $\pm$ 0.66
Ceftazidime hydrate (1 $\mu$ M)	2.15 $\pm$ 0.07*	0.95 $\pm$ 0.07	1.30 $\pm$ 0.04*	4.40 $\pm$ 0.11
Cinnamaldehyde (250 $\mu$ M)	1.84 $\pm$ 0.25*	1.58 $\pm$ 0.08*	0.89 $\pm$ 0.14*	4.30 $\pm$ 0.30
“Compound 3” (500 $\mu$ M)	2.46 $\pm$ 0.34*	1.18 $\pm$ 0.23	0.85 $\pm$ 0.19*	4.49 $\pm$ 0.46
Farnesol (2500 $\mu$ M)	2.44 $\pm$ 0.79*	1.29 $\pm$ 0.10*	0.49 $\pm$ 0.31*	4.22 $\pm$ 0.85
Indole (312 $\mu$ M)	1.79 $\pm$ 0.01*	0.96 $\pm$ 0.09	1.32 $\pm$ 0.11*	4.15 $\pm$ 0.15

3  
4

5

1 Figure 1:

2