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Evaluation of the effects of selected phytochemicals on quorum sensing inhibition and *in vitro* cytotoxicity

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10 Quorum sensing (QS) is an important regulatory mechanism in biofilm formation and differentiation. The interference with QS can affect biofilm development and antimicrobial susceptibility. This study evaluates the potential of selected phytochemical products to inhibit QS. Three isothiocyanates (allylisothiocyanate – AITC, benzylisothiocyanate – BITC and 2-phenylethylisothiocyanate – PEITC) and six phenolic products (gallic acid – GA, ferulic acid – FA, caffeic acid – CA, phloridzin – PHL, (−) epicatechin – EPI and oleuropein glucoside – OG) were tested. A disc diffusion assay based 15 on the pigment inhibition of *Chromobacterium violaceum* CV12472 was performed. In addition, the mechanisms of QS inhibition (QSI) based on the modulation of *N*-acyl homoserine lactones (AHLs) activity and synthesis by the phytochemicals were investigated. The cytotoxicity of each product was tested on a cell line of mouse lung fibroblasts. AITC, BITC and PEITC demonstrated capacity for QSI by modulation of AHLs activity and synthesis, interfering with QS systems of *C. violaceum* CviI/CviR homologs of LuxI/LuxR systems. The cytotoxic assays demonstrate low effects 20 on the metabolic viability of fibroblast cell line only for FA, PHL and EPI.

Keywords: biofilms; *Chromobacterium violaceum*; cytotoxicity; isothiocyanates; phenolics; phytochemicals; quorum sensing inhibition

Introduction

The emergence of multi-resistant bacteria to conventional 25 antibiotics has increased worldwide (Levy 2001; McDer- AQ1 mott et al. 2003; Andersson & Hughes 2010). This problem is apparently due to the natural selective pressure and to the indiscriminate use of antibiotics (Monroe & AQ2 Polk 2000; Andersson 2003). Another disadvantage of 30 the use of conventional antimicrobials is their failure to treat infections caused by bacterial biofilms (Costerton AQ3 et al. 1999; Anderson & O'Toole 2008). Bacteria in sessile state are more protected against host defenses, and highly tolerant to antimicrobials compared to their plank- 35 tonic counterparts (Lewis 2001). Consequently, new strategies are required to target those pathogenic microorganisms that are able to grow in biofilms (Landini et al. 2010; Saleem et al. 2010; Jorge et al. 2012). The best strategy to control biofilms is by preventing their devel- 40 opment. Therefore, the cellular processes of biofilm formation, maintenance, and dispersal are important targets for the discovery of new inhibitors (Landini et al. 2010). One of these approaches involves the use of compounds that interrupt the bacterial communication in biofilms, 45 instead of simply killing the bacteria (Kaufmann et al. 2008; Lee et al. 2011; Jakobsen et al. 2012).

Several Gram-negative pathogens use signaling molecules called autoinducers (AIs), such as *N*-acyl homoserine lactones (AHLs), to mediate communication systems in a process termed quorum sensing (QS) (Adonizio 50 et al. 2006). The first signal molecule of this type was identified from *Vibrio fischeri*, a marine symbiotic bacterium, which induces luminescence in this species (Dickschat 2010). These signaling molecules are synthesized by enzymes of the LuxI family (referred as AHL 55 synthethases), and can bind to transcription regulators of the LuxR family (Greenberg 2000). AIs are constantly being produced and received at basal level by bacterial cells (Adonizio et al. 2006; Dobretsov et al. 2013). When the cell density of a bacterial population increases, 60 the AHLs concentration also increases. The AIs produced by bacteria diffuse out and accumulate in the surrounding environment, and once a threshold concentration has been reached (quorum level), they diffuse back into the bacteria, and induce the expression of a set 65 of target genes, that consequently change the behavior of bacteria (Daniels et al. 2004). Hence, using QS bacterial populations can change from acting as individual cells to functioning in a concerted multi-cellular manner, controlling many physiological functions, such as 70

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bioluminescence, pigment production, production of antimicrobial compounds, conjugation, bacterial motility, and exopolysaccharide (EPS) synthesis (Whitehead et al. 2001; Manefield et al. 2002; Kaufmann et al. 2008; Dobretsov et al. 2011). Moreover, it has been shown that QS is an important regulatory mechanism in biofilm formation/differentiation and in the expression of genes involved in processes related to survival, virulence, and pathogenicity (Hentzer & Givskov 2003; Dickschat 2010; Landini et al. 2010). Various pathogenic bacteria such as *Pseudomonas aeruginosa*, *Vibrio* spp., *Burkholderia cepacia*, and *Yersinia enterocolitica* employed QS to regulate their virulence and pathogenicity (Khan et al. 2009).

The understanding of the mechanisms underlying biofilm formation could lead to approaches for their control and prevention. Biofilm formation is a dynamic and multicellular process mediated by a combination of adhesion mechanisms, EPS synthesis, bacterial motility, and QS phenomenon that are intrinsically related (Simões et al. 2009; Landini et al. 2010; Simões 2011). Several aspect of biofilm dynamic, such as heterogeneity, architecture, stress resistance, maintenance, and sloughing have been documented to be mediated by signaling molecules. The role of AHLs in the regulation of colonization events and in the differentiation of microcolonies was also described by Davies et al. (1998). Therefore, the interference with the communication system of microorganisms is a promising target to tackle biofilms (Zhang & Dong 2004; Landini et al. 2010). The discovery that several products with antibiofilm activity (eg halogenated furanones from Australian macroalgae, *Delisea pulchra*) are QS inhibitors demonstrated the importance of this signaling system in biofilm prevention and control (Hentzer et al. 2002; Manefield et al. 2002; Persson et al. 2005; Rasmussen, Skindersoe, et al. 2005). Moreover, other studies verified that with the block of signaling molecules, it is possible to reestablish the action of the immune system and, therefore, eliminate the infectious microorganism (Hentzer & Givskov 2003; Hentzer, Wu, et al. 2003; Bjarnsholt et al. 2005). Biofilms constitute not only a medical problem but may also have negative implications for the industry (food spoilage, pressure drop, biocorrosion, etc) and the environment (aquaculture diseases, drinking water distribution systems, etc). This microbial consortium is considered to be an initial step in the development of biofouling, which develops in practically all natural and engineered aqueous systems. Biofouling is a serious global problem in marine systems, causing extensive material and economic costs worldwide. The interference with bacterial QS has been proposed as one approach for controlling biofouling, due to the fact that compounds with capacity for QSI, affect biofilm formation and have also implication in biofouling (Qian et al. 2009, 2013). Natural products from marine organisms, fungi, and aquatic/terrestrial plants have shown to be effective inhibitors of biofouling due to their well-recognized capacity to produce QS inhibitors and, consequently, to inhibit biofilm formation (Dobretsov et al. 2009, 2011, 2013; Qian et al. 2009).

The majority of QSI compounds characterized so far have not been qualified as chemotherapeutic agents due to their toxicity, high reactivity, and instability (Khan et al. 2009). Hence, new products with less harmful effects have a greater advantage for humans, and the attention has been focused on identification of such compounds from natural and sustainable sources. In recent years, some reports have been published about natural compounds with capacity to inhibit QS, such as weeds, dietary products, and medicinal plant extracts (Gao et al. 2003; Vattem et al. 2007; Adonizio et al. 2008; Zhu & Sun 2008; Khan et al. 2009). Plants synthesize secondary metabolites (phytochemicals) that are a fundamental source of chemical diversity and important components of the current pharmaceutical products (Dixon 2001; Kubo et al. 2006; Saavedra et al. 2010; Saleem et al. 2010). In this context, it is known that some dietary phytochemicals, such as phenolics, glucosinolates, and their hydrolysis products, have beneficial health properties (Prior & Cao 2000; Aires, Mota, Saavedra, Monteiro, et al. 2009; Aires, Mota, Saavedra, Rosa, et al. 2009). These properties include, antibacterial, antiviral, antioxidant, anti-inflammatory, antiallergic and anticarcinogenic activities, hepatoprotective and antithrombotic effects, and vasodilatory action (Fahey et al. 2001; Soobrattee et al. 2005; Srinivasan et al. 2007; D'Antuono et al. 2009; Wang et al. 2010). Phenolic products, including simple phenolics, their derivatives and complex flavonoids and tannins, are among the most important and abundant plant secondary metabolites (Morton et al. 2000; Manach et al. 2004). These compounds can be found in vegetable, fruits, chocolate, and beverages (Soobrattee et al. 2005). Another important group of phytochemicals, known for their health benefits, are the glucosinolates hydrolysis products (GHP), particularly isothiocyanates (ITCs). These compounds can be found in the *Brassicaceae* family (ie cabbage, broccoli, mustard, horseradish and wasabi) and have long been recognized for their antimicrobial activity against clinical important microorganisms (Fahey et al. 2001; Kim & Lee 2009; Saavedra et al. 2010).

This study aims the assessment of the potential of nine selected phytochemicals (phenolic products and ITCs) in QS inhibition (QSI) and the evaluation of their cytotoxicity against a mouse lung fibroblast cell line in order to ascertain their potential application in antimicrobial chemotherapy. The selection of phytochemicals was based on previous studies performed with these compounds, which demonstrated good antimicrobial activity

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against Gram-negative and Gram-positive bacteria in both planktonic and sessile states (Saavedra et al. 2010; Borges et al. 2012; Borges, Ferreira, et al. 2013; Borges, Simões, et al. in press 2013).

5 **Materials and methods**

Bacterial strains and culture conditions

Chromobacterium violaceum CV12472 produces and responds to the AIs C6-AHL and C4-AHL, and was used to determine QSI activity. *C. violaceum* CV31532 10 (an overproducer of AIs C6-AHL) and CV026 (a mini Tn-5 mutant of the wild strain CV31532, which is not capable to produce its own AHL molecules, but responds to exogenous active signal molecules C4-AHL and C6-AHL) were used to evaluate the modulation of AHLs 15 activity and synthesis (McClean et al. 1997; Choo et al. 2006). The bacteria were routinely cultured aerobically in Luria–Bertani broth (LB; Liofilchen, Italy) at 30 °C with 150 rpm of agitation in a shaking incubator (AGITORB 200, Aralab, Portugal), prior to experiments. 20 LB agar was used to test the activity of phytochemicals on QSI and on modulation of AHL activity/synthesis.

Phytochemicals

The phytochemicals selected for screening of QSI AQ28 (Table 1), were: gallic acid (GA), ferulic acid (FA), caf- 25 AQ29 feic acid (CA), phloridzin (PHL), (−) epicatechin (EPI), oleuropein glucoside (OG), allylisothiocyanate (AITC), benzylisothiocyanate (BITC), and 2-phenylethylisothiocyanate (PEITC). All phytochemicals were obtained from Sigma-Aldrich (Portugal), and their solutions were pre- 30 pared with dimethyl sulfoxide (DMSO; Sigma, Portugal). Controls were performed with DMSO and LB broth.

Bioassay for detection of QSI

Standard disc diffusion assay (Bauer et al. 1966) was performed with biosensor strain *C. violaceum* CV12472 35 to detect QSI activity of the selected phytochemicals. Concentrations lower, equal, and higher than the MIC (minimum inhibitory concentration) (5, 10, 15, 20, 25, 50, 75, 100, 112.5, 150, 200, 250, 300, 350, 375, 500, 750 and 1,000 μg ml^{-1}), that were previously determined 40 by the microdilution method (Borges et al. 2012), were tested. Briefly, LB agar (LB; Liofilchen, Italy) plates were inoculated with 100 μl (1.4×10^8 CFU ml^{-1}) from an overnight culture of *C. violaceum* CV12472. Afterwards, sterile paper disks (6 mm in diameter) (Oxoid, 45 Spain) were placed over the plates and were loaded with 15 μl of different concentrations of each phytochemical. DMSO and LB broth were used as controls. In the biosensor strain *C. violaceum* CV12472, the production of a purple pigment (violacein) is under control of a QS 50 system that in turn is mediated by the activity of AHLs (McClean et al. 1997; Chernin et al. 1998; Stauff & Bassler 2011). These signaling molecules are produced AQ9 by the autoinducer synthase CviI, and when a quorum has been reached, the AHLs bind to the transcriptional regulator CviR and this complex triggers the expression 55 of violacein production (Choo et al. 2006). After 24 h of incubation at 30 °C, the inhibition of the pigment production around the disc (a ring of colorless but viable cells) was checked. Antimicrobial activity is indicated by the lack of microbial growth. Bacterial growth inhibition 60 was measured as diameter 1 (d_1) in mm while both bacterial growth and pigment inhibition were measured as a total diameter 2 (d_2) in mm. Thus, QSI, assessed by pigment inhibition, was determined by subtracting the diameter of bacterial growth inhibition (d_1) from the total 65 diameter (d_2) ($QSI = d_2 - d_1$), according to Zahin et al. (2010).

After the initial screening, using the qualitative agar discs diffusion method, QSI caused by phytochemicals was also quantified in a broth assay, according to Choo 70 et al. (2006). With this purpose, *C. violaceum* CV12472 ($OD_{620\ nm} = 0.1$) supplemented with different concentrations of phytochemicals were incubated at 30 °C in a shaking incubator (150 rpm) for 24 h. Then, violacein extraction was carried out. Briefly, 1 ml of culture from 75 each flask was centrifuged at 14,549 *g* for 10 min to precipitate the insoluble violacein and bacterial cells. The culture supernatant was discarded and 1 ml of DMSO was added to the pellet. The solution was vortexed vigorously for 30 s to completely solubilize violacein, and 80 centrifuged at 14,549 *g* for 10 min to remove the cells. Two hundred microlitres of the violacein containing supernatants were added to 96-well polystyrene microtiter plates and the absorbance was read with a microplate reader (Spectramax M2e, Molecular Devices, Inc.) at a 85 wavelength of 585 nm. The results were expressed as percentage of violacein reduction. All tests were performed in triplicate with three independent experiments.

Modulation of AHLs activity and synthesis

The agar diffusion double ring assay was used to evalu- 90 ate the effect of the phytochemicals on the modulation of both AHLs activity and synthesis, as described previously (McLean et al. 1997). For this test, only the phytochemicals that showed capacity for QSI in the disc diffusion assay were used. After overnight growth, the 95 cultures of *C. violaceum* CV31532 and *C. violaceum* CV026, were adjusted to $OD_{620\ nm} = 0.1$ and subsequently assayed for violacein production. Sterile paper disks were placed on the center of the LB agar plates and were loaded with 15 μl of different phytochemical 100 concentrations. Controls with DMSO and LB broth were performed. To test for AHLs activity, the biosensor

Table 1. Subgroups of phenolic and glucosinolate compounds, their chemical structure, dietary source, biological properties, and MIC values against *C. violaceum* CV12472.

Phytochemical name	Chemical class	Chemical structure	Dietary source	Biological properties	References	MIC (μg ml^{-1})
Gallic acid (GA) (3,4,5-trihydroxybenzoic acid)	Phenolic acid (hydroxybenzoic acid)		Grapes (grape seeds), berries, tea leaves, oak bark	Antioxidant Antibacterial		NS
Ferulic acid (FA) (4-hydroxy-3-methoxy cinnamic acid)	Phenolic acid (hydroxycinnamic acid)		Cereals (corn flour, grain wheat, rice and oat flours).	Anti-inflammatory		NS
Caffeic acid (CA) (3,4-dihydroxycinnamic acid)	Phenolic acid (hydroxycinnamic acid)		Many fruits and vegetables (coffee)	Antiallergic		NS
Phloridzin (PHL) (3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenyl β-D-glucopyranoside)	Flavonoids (Chalcone glucoside)		Apples	Hepatoprotective; Antithrombotic	Cushnie and Lamb (2005), Soobrattee et al. (2005), Boudet (2007), Srinivasan et al. (2007), and Cueva et al. (2010)	NS
(-) Epicatechin (EPI) (2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzo- pyran-3,5,7-triol)	Flavonoids (flavanols)		Many fruits, cocoa, nuts.	Antiviral		NS
Oleuropein Glucoside (OG) (2-(3,4-Dihydroxyphenyl)ethyl [2S-(2alpha,3E,4beta)]-3-ethylidene-2-(beta-D-glucopyranosyloxy)-3,4-dihydro-5-(methoxycarbonyl)-2H-pyran-4-acetate)	Flavonoids (*seco*-iridoid)		Olives (bark and leaves).	Anticarcinogenic; Vasodilator		NS
Allylisothiocyanate (AITC) (3-Isothiocyanato-1-propene)	GHP (isothiocyanate)		*Brassica* Species (cabbage, broccoli, cauliflower).	Antimicrobial	Fahey et al.(2001), Shin et al. (2004), Hong and Kim (2008), D'Antuono et al. (2009), Saavedra et al. (2010), Wang et al. (2010), and Dufour et al. (2012)	15
Benzylisothiocyanate (BITC) (Isothiocyanatomethylbenzene)	GHP (isothiocyanate)		Garden cress and *Brassica* Species.	Antioxidant		10
2-Phenylethylisothiocyanate (PEITC) (2-Isothiocyanatoethylbenzene)	GHP (isothiocyanate)		Watercress and *Brassica* species.	Anticarcinogenic		15

(NS) – Non-susceptible, the MIC is higher than the maximum concentration tested (>1,000 μg ml^{-1}).

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C. violaceum CV026 was streaked in a circle on the LB agar plates in close proximity to the tested phytochemical and the overproducer *C. violaceum* CV31532 was streaked on the outside (4–5 mm of distance between two biosensors). To assess the inhibition of AHLs synthesis, the location of AHLs overproducer and biosensor strains was inverted. Three independent assays were performed.

Cell line and culture conditions

The cell line of mouse lung fibroblasts (L929), obtained from the European Collection of Cell Cultures (ECACC) was selected for cytotoxicity studies. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma, Portugal), supplemented with 10% of fetal bovine serum (FBS; Biochrom, Germany), 1% of antibiotics penicillin–streptomycin (PEN-STREP; Invitrogen), and 3.7 g l^{-1} of sodium bicarbonate, and incubated at 37 °*C* in a humidified atmosphere containing 5% of CO_2/95% air, prior to the experiments.

Cytotoxicity screening

In order to evaluate the *in vitro* cytotoxicity of the selected phytochemicals, the cells were grown in DMEM with FBS and antibiotics (penicillin and streptomycin, Sigma), and trypsinized before the experiments, according to ISO/EN 10993 (part 5) guidelines with some modifications (ISO document 10993 1992). Then, the cell line was seeded at an appropriate density (1×10^4 per cells well) in 96-well polystyrene microtiter plates (5 wells per tested condition) and allowed to attach for 24 h at 37 °C, in a humidified atmosphere containing 5% of CO_2. After 24 h of cell seeding, culture medium was discarded and replaced by 200 µl of fresh DMEM with each phytochemical at several concentrations (50, 100, 500, and 1,000 µg ml^{-1}) that were prepared with DMSO, and incubated for 72 h at 37 °C in a humidified atmosphere containing 5% of CO_2. The phytochemicals did not exceed 1.5% (v/v) of the well final volume. Cells with DMSO (1.5%, v/v) and without phytochemicals were used as controls. The cell viability was evaluated by MTS assay (Baran et al. 2004). Three independent experiments were performed.

Cell viability assay

After 72 h of exposure to phytochemicals, cell viability was assessed using Cell Titer 96® One solution Cell Proliferation Assay kit (Promega, USA). In this test, the substrate MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is bioreduced into a brown formazan product by dehydrogenase enzymes in metabolically active cells (Baran et al. 2004). The phytochemicals were removed with a micropipette and 200 µl of MTS solution, prepared with DMEM with antibiotics and without FBS in a 5:1 ratio, was added into each well. Cells were then incubated for 3 h at 37 °C in a humidified atmosphere containing 5% of CO_2 in the dark. The optical density of each well was measured at 490 nm using a microplate reader. The results were expressed as percentage of cell viability.

Statistical analysis

The data were analyzed using the statistical program SPSS version 20.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. At least three independent experiments were performed for each condition tested. One-way Anova with Bonferroni test was used to assess the statistical significance value (confidence level ≥95%). $p < 0.05$ was considered statistically significant.

Results

The MICs of the tested phenolics and ITCs against biosensor strain *C. violaceum* CV12472 are presented in Table 1. The MIC of all phenolics tested was >1,000 µg ml^{-1}. BITC had the lowest MIC (10 µg ml^{-1}) while AITC and PEITC had a MIC of 15 µg ml^{-1}.

A disc diffusion assay was performed for QSI screening using the biosensor strain *C. violaceum* CV12472. A range of concentrations (from sub-MICs to >MICs) were tested in order to ascertain if the halos produced around the disks were due to growth inhibition of cells and/or QSI (see Supplementary material). Hence, loss of purple pigment by *C. violaceum* CV12472 is indicative of QSI by the phytochemicals. Amongst the nine phytochemicals screened, no pigment inhibition was observed with phenolics at the tested concentrations. However, growth inhibition halos between 8 and 12 mm were detected (data not shown). GA and FA at concentrations between 5 and 100 µg ml^{-1} produced halos of growth inhibition of 8 mm and, for higher concentrations, the halos size was around 11 and 12 mm. Other phenolics, CA, OG, PHL, and EPI, produced halos of growth inhibition of 10 mm for all the concentrations tested (data not shown). QSI was only detected for the three ITCs (Table 2). AITC at concentrations between 5 and 200 µg ml^{-1} caused halos of growth inhibition of 10 and 11 mm, and no activity on pigment inhibition was observed. The concentrations of 250, 300, and 350 µg ml^{-1} caused partial pigment reduction in addition to antimicrobial activity (halos of 14 mm). AITC caused QSI halos of 5 (350 µg ml^{-1}), 14 (375 and 500 µg ml^{-1}), and 45 mm (750 and 1,000 µg ml^{-1}). For these cases, the white zones of inhibition were opaque and not transparent, indicating that the halo observed was caused by

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Table 2. Screening of ITCs that exhibited QSI and antimicrobial activity against *C. violaceum* strain CV12472.

Phytochemical	AITC			BITC			PEITC		
μg ml^{-1}	Pigment production	AM[a] (mm)	QSI[b] (mm)	Pigment production	AM[a] (mm)	QSI[b] (mm)	Pigment production	AM[a] (mm)	QSI[b] (mm)
Medium	+	0	0	+	0	0	+	0	0
DMSO	+	0	0	+	0	0	+	0	0
5	+	10	0	+	10	0	+	11	0
10	+	10	0	+	12	0	+	11	0
15	+	11	0	+	20	8	+	15	0
20	+	10	0	+	20	8	+	15	0
25	+	11	0	+	20	8	+	15	0
50	+	10	0	+	20	8	+	22	0
75	+	10	0	+	54	6	+	27	0
100	+	10	0	+	52	13	+	30	0
112,5	+	11	0	+	65	12	+	33	0
150	+	11	0	+	65	10	+	33	0
200	+	11	0	+	74	5	+	33	0
250	+/−	14	0	+	74	5	−	35	5
300	+/−	14	0	t.i	t.i	t.i	−	40	10
350	+/−	14	5	t.i	t.i	t.i	−	40	10
375	−	20	14	t.i	t.i	t.i	−	40	30
500	−	24	14	t.i	t.i	t.i	−	40	30
750	−	40	45	t.i	t.i	t.i	−	50	35
1000	−	40	45	t.i	t.i	t.i	−	50	35

(+)-visualization of pigment color; (−)-absence of pigment color; (+/−)-partial visualization of pigment color; (t.i) – total inhibition (without grow).
[a]Antimicrobial activity observed as a clear halo, presented as diameter of zone of inhibition in millimeter.
[b]QSI observed as a halo of colorless with viable cells, presented as diameter of QSI in millimeter.

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inhibition of violacein production, and not due to cell growth inhibition. Moreover antimicrobial activity was also observed in addition to QSI, producing halos of growth inhibition around the disks of 14 (350 μg ml^{-1}), 20 (375 μg ml^{-1}), 24 (500 μg ml^{-1}), and 40 mm (750 and 1,000 μg ml^{-1}) of diameter (Table 2). For BITC at 5 and 10 μg ml^{-1}, no pigment inhibition was observed, but it was detected antimicrobial activity with halos of 10 and 12 mm, respectively (Table 2). For concentrations between 15 and 250 μg ml^{-1}, it was observed antimicrobial activity (inhibition zones of 20–74 mm) and also QSI (halos ranging from 5 to 13 mm). For concentrations higher than 250 μg ml^{-1}, the growth of the biosensor strain was completely inhibited. For PEITC, clear halos indicating the absence of growth of the biosensor microorganism were observed for concentrations between 5 and 200 μg ml^{-1}. For all concentrations higher than 200 μg ml^{-1}, both QSI (halos ranging from 5 to 35 mm) and growth inhibition (inhibition halos of 35–50 mm) were observed.

In order to evaluate the extent of QSI, the extraction and quantification of violacein from CV12472 cultures in the absence and presence of phytochemicals at different concentrations were also performed. Figure 1 shows the results of violacein inhibition by ITCs (%). Violacein production was inhibited more than 85% with AITC for all concentrations tested, except for the concentration of 5 μg ml^{-1} (70%). For concentrations between 10 and 1,000 μg ml^{-1}, no increase of violacein inhibition was observed ($p < 0.05$). The same behavior was verified with BITC. For concentrations higher than 15 μg ml^{-1}, the percentage of violacein inhibition was around 90%. At 5, 10, and 15 μg ml^{-1}, the percentage of violacein inhibition with BITC was 81, 84, and 86%, respectively. For PEITC, violacein production was inhibited more than 80% for concentrations higher than 75 μg ml^{-1}. A maximum of 89% inhibition in violacein production was observed with PEITC at 1,000 μg ml^{-1} (Figure 1). For the smaller concentrations, the percentage of violacein inhibition was 47, 57, 60, 76, 77, and 79%, for concentrations of 5, 10, 15, 20, 25, and 50 μg ml^{-1}, respectively. Since QSI was not observed with the phenolic compounds by the disk diffusion method, only the highest concentration used was selected for broth assays. The percentage of violacein inhibition by phenolics at 1,000 μg ml^{-1} was: 75 (CA), 72 (FA), 59 (GA), 51 (OG), 48 (PHL), and 33% (EPI) (Figure 2).

The effects of ITCs (which had positive results for QSI) on modulation of AHLs activity and synthesis was also performed. For this assay, a range of concentrations above and below the one that inhibited the violet pigment were selected. The AHLs activity was assessed by the decrease of the violacein pigment production in *C. violaceum* CV026 AHL biosensor, due to low levels

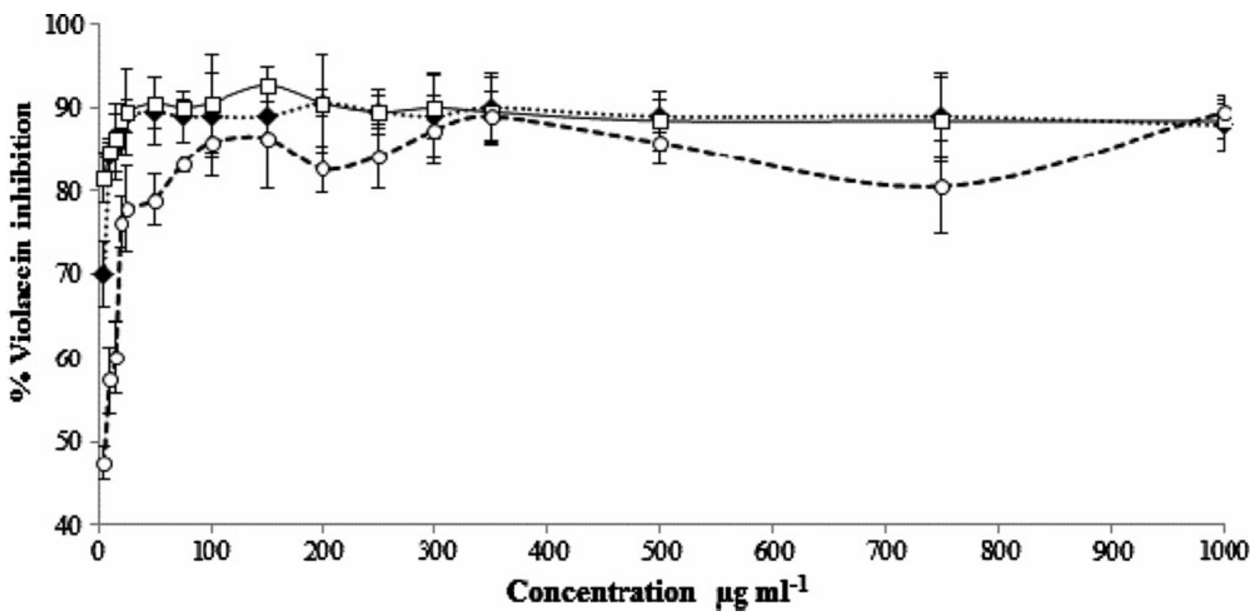

Figure 1. Percentage of violacein inhibition (%) by ITCs, AITC (♦), BITC (□), and PEITC (○) at different concentrations (5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 300, 350, 500, 750, and 1,000 µg ml^{-1}). Mean values ± standard deviation for at least three replicates are illustrated.

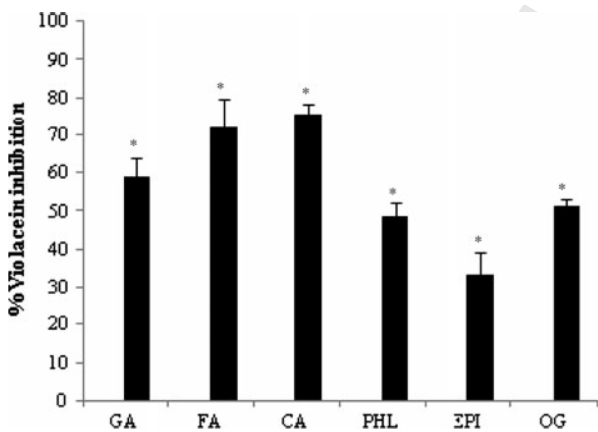

Figure 2. Percentage of violacein inhibition (%) by phenolic compounds (GA, FA, CA, PHL, EPI, and OG) at 1,000 µg ml^{-1}. Mean values ± standard deviation for at least three replicates are illustrated. Bars with * are statistically different from the control ($p < 0.05$).

of QS related with AHL detection. The AHLs synthesis was also evaluated by pigment inhibition of CV026, due to the decrease or absence of AHLs production by over-producer *C. violaceum* CV31532 in the presence of the phytochemicals. Our results demonstrated that AITC had capacity to interfere with the activity of the AHLs produced by CV31532. This effect started at 25 µg ml^{-1}, causing a small decrease in the violacein production which was intensified at 100 and 250 µg ml^{-1} (data not shown). No pigment was observed for concentrations higher than 350 µg ml^{-1} (data not shown). For BITC, pigment inhibition of CV026 was verified for concentrations between 2 and 5 µg ml^{-1} (data not shown). Total inhibition was observed for the higher concentrations. PEITC also showed positive interference with AHLs activity at 20 µg ml^{-1} and no pigment of CV026 was found for concentrations above 50 µg ml^{-1} (data not shown). The results on the ability of ITCs to reduce the production of AHLs molecules from CV31532 were identical to AHLs activity (data not shown).

The cytotoxicity of the selected phytochemicals was tested on a cell line of mouse lung fibroblasts (L929) (Figure 3). L929 cells produced large amounts of a brown formazan product after 72 h of exposure to PHL for all concentrations tested. The viability after exposure to this phenolic was statistically equal to the control ($p > 0.05$) except for the concentration of 1,000 µg ml^{-1} ($p < 0.05$). FA at 50 and 100 µg ml^{-1} also produced higher quantity of formazan with a percentage of viable cells of 98 and 95%, respectively ($p > 0.05$). Viabilities of 96 and 95% were obtained after exposure to EPI at 50 and 100 µg ml^{-1}, respectively. With these compounds at 500 µg ml^{-1} (FA – 67%; EPI – 17%) and 1,000 µg ml^{-1} (FA – 28%; EPI – 30%), it was verified a decrease in the percentage of viable cells ($p < 0.05$). The percentage of viable cells with OG at 50 µg ml^{-1} was 57%. The increase of OG concentration reduced significantly the cells viability ($p < 0.05$). The ITCs reduced significantly the viability of the L929 cells

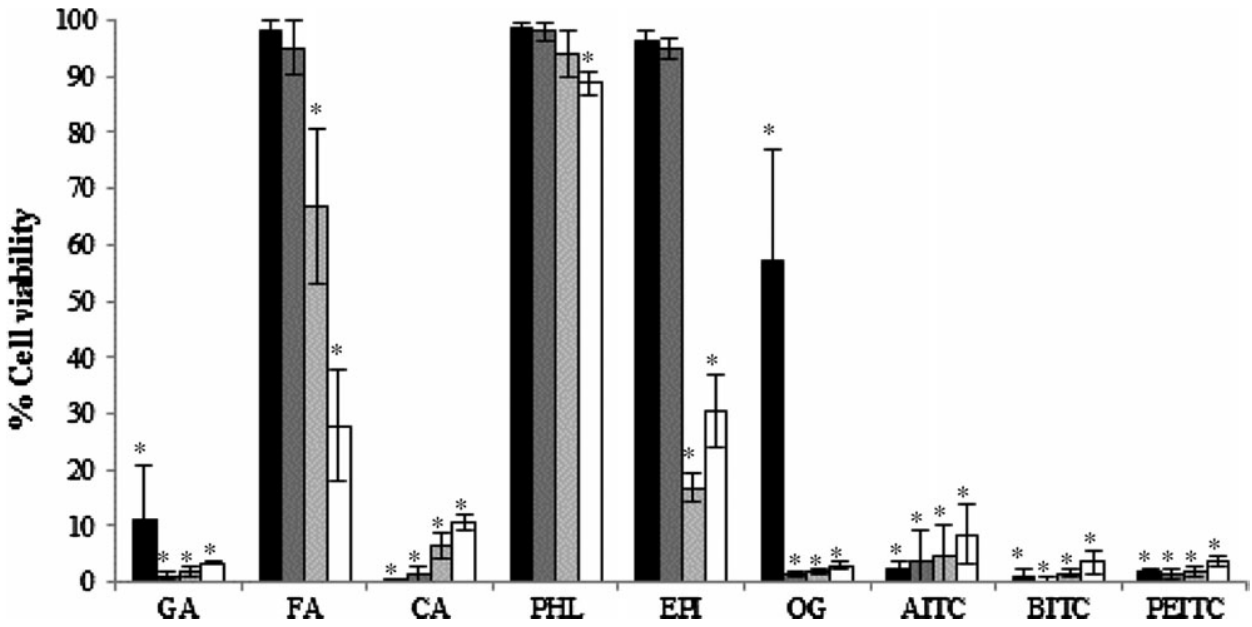

Figure 3. Percentage of viability (%), obtained with the MTS assay, of the L929 cells exposed to different concentrations (50 (■), 100 (■), 500 (■), and 1,000 (□) μg ml^{-1}) of phenolic compounds (GA, FA, CA, PHL, EPI, and OG) and ITCs (AITC, BITC, and PEITC) for a period of 72 h. Mean values ± standard deviation for at least three replicates are illustrated. Bars with * are statistically different from the control ($p < 0.05$).

($p < 0.05$), being the toxicity levels similar for the diverse concentrations tested ($p > 0.05$). The same behavior was verified with GA and CA ($p < 0.05$).

Discussion

The occurrence of planktonic and sessile bacteria, resistant to antimicrobials, increases the need for the development of new strategies to control microbial growth (Adonizio et al. 2006; Khan et al. 2009). More knowledge about QS systems allowed the employment of new approaches for the development of drugs to control bacterial infections by different mechanisms from that of actual antibiotics. So, the disruption of the QS signaling pathways can help to overcome the bacterial resistance problem and is a possible key to treat infections caused by biofilms (Dickschat 2010; Zhu et al. 2011; Jakobsen et al. 2012; Qian et al. 2013).

In the present study, phenolic compounds and ITCs, that are commonly present in dietary products, were evaluated as potential QS inhibitors. These compounds are thought to be an integral part of both human and animal diets. Preliminary reports showed that some of these phytochemicals have antimicrobial properties with potential for biofilm prevention and control (Borges et al. 2012; Borges, Simões, et al. in press 2013).

The results of the qualitative screening of nine phytochemicals indicated that all ITCs tested (AITC, BITC, and PEITC) have potential QSI activity based on the production of *C. violaceum* pigment. In fact, ITCs had antimicrobial activity in addition to QSI. For these compounds, two halos were observed: the first was a clear halo (bacterial growth inhibition), demonstrating antimicrobial activity; the second was opaque (bacterial growth without violet pigment production) and corresponds to QSI. Similar results were observed in other studies, with extracts and essential oils from medicinal plants (Adonizio et al. 2006; Khan et al. 2009; Koh & Tham 2011). This QSI may be associated to the AQ10 antimicrobial activity of phytochemicals, as proposed by Skindersoe et al. (2008)

The analysis of the ITCs structure and their QSI shows a higher potential of the aromatic GHP (BITC and PEITC) relatively to aliphatic GHP (AITC). In addition, BITC was the most effective ITC for QSI, acting at low doses (15–250 μg ml^{-1}). However, the QSI activity verified with the ITCs tested needs to be further characterized regarding the genes that are affected by the phytochemicals.

The QS systems of *C. violaceum* consist of CviI/CviR that are homologs of LuxI/LuxR systems. This strain mediates QS by AIs of the type of AHLs (C4-AHL and C6-AHL) (Blosser & Gray 2000; Morohoshi et al. 2008; Stauff & Bassler 2011). AQ11 These molecules are AHL-based QS systems synthesized by LuxI-type AHL synthases and detected by their cognate LuxR-type receptors. Based on the fact that the ITCs tested in this study inhibit QS systems of *C. violaceum*, and for the

high similarity of QS systems that use the same type of AHLs signaling molecules (Dickschat 2010), it is possible that these compounds can interfere with other homologues QS systems. Additional studies with other biosensors that use different QS systems are need to confirm this possibility.

Phenolic compounds inhibited the growth of the bacterium but not the pigment production. The lack of QSI detection of the tested phenolics can be due to the limitations of QSI assay used. These compounds can affect QS systems in a different way from that of *C. violaceum.* Indeed, Huber et al. (2003) found that some polyphenolic compounds having a GA moiety [(−)-epigallocatechin gallate, (+)-catechin and tannic acid] shown positive interference with bacterial QS of *Escherichia coli* and *Pseudomonas putida*. Liang et al. (2009) observed that salicylic acid showed anti-QS activity against *P. aeruginosa* in a dose-dependent manner. Extracts from *Moringa oleifera*, which contains phenolics compounds such as GA, chlorogenic acid, and quercetin in appreciable quantities demonstrated QSI potential (Singh et al. 2009).

The common mechanisms of QS interference include: (i) inhibition of signal biosynthesis or inhibition of activity of AHLs-producing enzymes, (ii) enzymatic signal degradation, and (iii) inhibition of reception signal molecules (Zhang & Dong 2004; Rasmussen & Givskov 2006a, 2006b; Khan et al. 2009). In this study, it was investigated the effects of ITCs on modulation of AHLs activity (*via* LuxR-type receptor) and synthesis (*via* LuxI-type AHL synthases). All ITCs revealed capacity to modulate both AHLs activity and synthesis. The results of CV31532/CV026 systems also indicate that BITC was the most potent ITC based on pigment inhibition of CV026 strain. Based on both results of disc diffusion and double ring assays, it is possible to infer that QSI in the *C. violaceum* systems, with ITCs, can be mediated by the interference with synthesis or activity of AIs C6-AHLs. Therefore, QSI can be due to a combination of two mechanisms: interference with the activity of AHLs produced by CV31532 (LuxR system homologue) and modulation of the synthesis of AHLs by CV31532 (LuxI system homologue) as detected by the extension of violacein production by the AHLs biosensor CV026. This result was also found by Vattem et al. (2007) with dietary phytochemicals. Chenia (2013) achieved the same result with *Kigelia africana* extracts.

QSI by ITCs can be achieved by several ways: the ITCs could affect the QS signaling cascade of CV31532 strain, binding directly to the LuxR-type receptor by competing with the AHLs molecules and/or by preventing the binding of the AHLs molecules to these receptors (Hentzer & Givskov 2003; Rasmussen, Bjarnsholt, et al. 2005; Rasmussen, Skindersoe, et al. 2005). These phytochemicals could also affect the ability of CV31532 to synthesize AHLs by decreasing the expression of the LuxI-type synthase, which synthesizes the AHLs molecules. Decreased AHLs synthesis may also be explained by the ability of these phytochemicals to inhibit the LuxI-type enzyme activity (Schauder & Bassler 2001). Since the ITCs were effective at inhibiting QS mediated by two different AHLs producers (CV12472 and CV31532), it may be assumed that these compounds are able to inhibit multiple bacterial QS systems homologues of LuxI/LuxR systems which are mediated by AHLs molecules.

Although the disc diffusion method is effective for the detention of inhibitors of QS, it is not possible to know the exact quantities of violacein inhibition. This drawback indicates the need for an assay that can measure the amount of violacein production. With the aim of reinforcing the results obtained in the qualitative assay related to pigment inhibition, violacein was extracted and quantified, after 24 h of exposure to phytochemicals. The results showed a percentage of violacein inhibition higher than 70% for ITCs regardless the concentration used, except for PEITC at the lowest concentrations (5, 10, and 15 μg ml^{-1}). The results obtained by broth studies also demonstrated significant inhibition of the percentage of violacein production by phenolic compounds. Although, in disc diffusion assay, no positive result for QSI was observed. This is apparently due to inhibition of microbial growth and not violacein synthesis. Another possibility for the observed results with the phenolics is the alteration of the permeability of the cell membrane that influence the flux of AHLs, as verified by Skindersoe et al. (2008). In fact, phenolic compounds, such as GA and FA, can interfere with the membrane permeability of some Gram-negative and Gram-positive bacteria, as demonstrated in a previous study (Borges, Ferreira, et al. 2013).

The use of QS inhibitors could have the potential to circumvent the problem of bacterial resistance (Cady et al. 2012). Since reports on dietary phytochemicals as source of QS modulators are scarce, the results obtained in this work with ITCs are relevant. The presence of such compounds in natural foods is extremely interesting because, in most cases, vegetables are non-toxic to humans and readily available. Additionally, functional foods with beneficial effects for health have attracted the attention of researchers and can be used as a prophylactic treatment. Natural products with capacity for QSI can be used with antibiotics as adjuvants in order to increase the susceptibility of resistant bacteria (Cady et al. 2012). It was demonstrated by Brackman et al. (2011) that QSI increased the susceptibility of bacterial biofilms to multiple types of antibiotics.

In order to know whether phenolic compounds or ITCs constitute pharmaceutically relevant drug candidates, it was investigated the cytotoxic effects on

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mammalian cells, using a cell line L929 that is routinely used in, *in vitro*, cytotoxicity assessments. The results showed that 72 h after contact with PHL (at all concentrations), FA (at 50 and 100 μg ml^{-1}), and EPI (at 50 and 100 μg ml^{-1}), the majority of cells are viable. This supports the absence of cytotoxic effects of these compounds and suggests their possible usage for therapeutic applications. The addition of 50 μg ml^{-1} of OG was sufficient to reduce about 50% of the cellular viability. Concentrations higher than 50 μg ml^{-1} significantly decreased the percentage of viable cells. The same result was observed with GA, CA and with the three ITCs used, for all the concentrations tested. It is important to refer that these compounds had a concentration-dependent effect on the viability of cells, except CA. The increase of CA doses had a positive effect on the viability of the cells, ie higher CA dose were less toxic to the cells. In fact, it is recognized that CA stimulates cell proliferation at high doses (Ito et al. 1993; Kagawa et al. 1993; Lutz et al. 1997). Although GA, FA, and CA are phenolic acids, these compounds demonstrated different cytotoxicity. This is apparently due to differences in their structure. The properties of phenolic products vary according to the type of substituents, and with the number and positions of the hydroxyl groups on the aromatic ring (Robbins 2003; Sroka & Cisowski 2003; Stalikas 2007). GA demonstrated to be more cytotoxic than CA and FA. This is probably due to the fact that GA has more hydroxyl groups than CA and FA and, consequently, can interact more strongly with the cells. In the same way, CA (with 2 hydroxyl groups) is more cytotoxic than FA (with only 1 hydroxyl group). Nevertheless, the relationship between the structural and cytotoxic differences of GA, FA, and CA needs to be further elucidated. In a work performed by Galati et al. (2006), it was verified that the major cytotoxic mechanism found by GA for hepatocytes was mitochondrial membrane potential collapse and reactive oxygen species (ROS) formation. Likewise, these cytotoxic effect and ROS formation was increased by the presence of some metals such as copper, and enzymes (NAD(P)H:quinone oxidoreductase 1). But these authors also demonstrated that formation of an iron complex and glutathione (GSH) conjugation play an important role in the detoxification of GA. So, the exposure of cells to GA in the presence of iron can reduce the cytotoxic effect of GA as verified in this study. However, more tests are needed to determine the additional aspects of the cytotoxicity of this compound.

The percentage of viable cells after the contact with the ITCs was low. It is known that the differences in the activity of ITCs are dependent of the intracellular uptake (Zhang 2000, 2001). GSH may be involved in the uptake of ITCs, occurring mainly in the form of GSH-conjugated dithiocarbamates, catalyzed by glutathione transferases (GST) (Zhang 2000, 2001). So, the accumulation of ITCs is related to intracellular GSH levels. Nevertheless, the degree of such synergism could depend on the type of ITC. For AITC, BITC, and PEITC, this synergism may be sometimes limited (Zhang 2001; Fimognari et al. 2004). Therefore, it is possible to hypothesize that the cytotoxicity observed in this study for these ITCs can be in part, but not totally, due to this mechanism. Moreover, their high cytotoxic effects may be attributed to the reactive of the ITC group, which can react with proteins and other crucial biomolecules. This reactivity can lead to the formation of oxygen and other free radicals that might cause cellular injuries. However, *in vitro* studies do not necessarily predict *in vivo* outcomes. It is known that ITCs are unstable and reactive in physiological environments. According to *in vivo* studies performed by Amara et al. (2009), the reactive ITC group may react with host-encoded proteins and therefore eliminate its bioactivity. Nevertheless, epidemiological studies indicate that consumption of cruciferous vegetables reduce the risk of diverse human cancers due to anticarcinogenic properties. There are a significant number of reports indicating that ITCs can exert a cytotoxic action against tumor cells perturbing several steps in the carcinogenic process (inhibition of cell growth, induction of apoptosis, DNA fragmentation, and cell death) (Xu & Thornalley 2000; Fimognari et al. 2004; Zhang 2010).

Although the cytotoxicity observed in this work with the ITCs tested, the European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to Food (ANS) concluded in a recent report about the safety of AITC for the use as a food additive, that no significant safety concerns are predictable with its use as anti-spoilage agent (EFSA 2010). Moreover, ITCs products are promising candidates for food industry due to their recognized antimicrobial activity against food-borne pathogens, and because they do not influence the organoleptic properties of processed food (Delaquis & Mazza 1995; Aires, Mota, Saavedra, Monteiro, et al. 2009; Al-Gendy et al. 2010). In a recent report of Mushantaf et al. (2012), AITC was suggested as a possible disinfectant for non-potable water treatment. The relatively immediate aqueous degradation of AITC is an advantage because it will not persist in the environment (Gómez De Saravia & Gaylarde 1998; Mushantaf et al. 2012). Based on these reports, AITC can be also a potential candidate to prevent biofouling and microbial diseases in aquaculture.

In conclusion, AITC, BITC, and PEITC were found to have QSI activity. These ITCs affected the QS system of *C. violaceum* (CviI/CviR system – LuxI/LuxR homolog) not only interfering with AHLs activity but also by modulating AHLs synthesis. These phytochemicals were highly cytotoxic for the cell line of mouse lung fibroblasts. The phenolic compounds tested did not showed

QSI potential, apparently due to the fact that they can interfere with other QS systems, not covered by the assay used in this study. The initial *in vitro* antimicrobial and cytotoxic tests suggest the potential use of FA, PHL, and EPI as therapeutic antimicrobials. These results are particularly important in the case of FA, due to its antimicrobial activity against strains with pathogenic potential in both planktonic and biofilm states (Borges et al. 2012; Borges, Ferreira, et al. 2013), and to potentiate the action of antibiotics (Saavedra et al. 2010).

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Supplemental material

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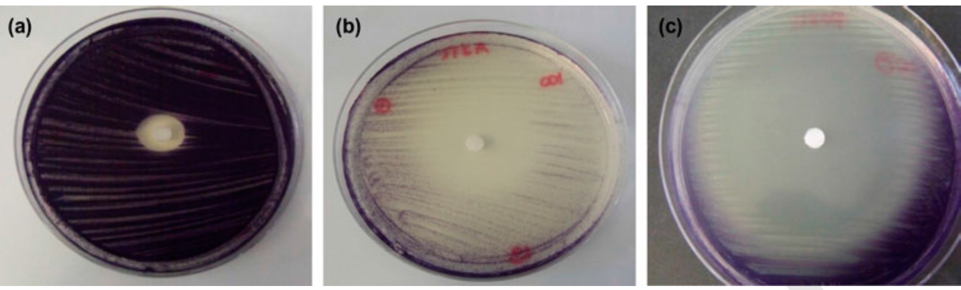

Figure S1. Disc diffusion assay using the biosensor strain *C. violaceum* (CV 12472) to evaluate the activity of ITCs on QSI. ITCs displayed different results: antimicrobial activity indicated by clear zones (inhibition of growth) (a); reduction of the violacein production indicated by partial visualization of pigment (b); and QSI indicated by a colorless halo (opaque zones) with viable cells (the bacteria were able to grow, but did not produce violacein). In b and c it is also possible to observe antimicrobial activity (clear halo around the disks), in addition to pigment inhibition.

Proof Only