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Anti-Quorum Sensing Activity of Natural Compounds against *Chromobacterium violaceum*

Abstract

The resistance and potential pathogenesis of bacteria could be related to their ability to sense and respond to population density, termed quorum-sensing (QS). Inhibition of the QS system is considered as a novel strategy for development of antipathogenic agents, especially for combating bacterial infections caused by antibiotic-resistant strains. In this work, the anti-QS activity of vanillin, geraniol and pomegranate extract was tested against *Chromobacterium violaceum*. Moreover, the minimum QS inhibitory concentration (MQSIC) was estimated for each agent. MQSIC's found were very low (0.60 mg/mL for vanillin; 0.19 µL/mL for geraniol; and 25 µg/mL for pomegranate extract). Therefore, the natural agents tested in this work are good candidates for the development of anti-QS compounds with potential application for the control of bacterial diseases regulated by QS systems.

Keywords: Cell-to-cell communication; Vanillin, Geraniol; Pomegranate extract; MQSIC

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Introduction

Despite advances in food safety, foodborne diseases still occur around the world. Each year in the United States, more than 9 million people suffer illnesses caused by major foodborne pathogens, such as *Escherichia coli* O157:H7, *Salmonella*, *Campylobacter*, and *Listeria monocytogenes* [1]. The resistance and potential pathogenesis of these bacteria could be related to their ability to sense and respond to population density. This ability is termed "cell-to-cell communication" or "quorum sensing" (QS), which is based on the synthesis, exchange and perception of small signaling molecules at given cellular densities [2]. These auto inducer molecules have been identified generally as oligopeptides in gram-positive bacteria and acylated homoserine lactones (AHLs) in gram-negative bacteria, but autoinducers also include butyrolactones, alkyl-quinolones and furanones [3]. These mechanisms allow bacteria to regulate some physiological activities, such as virulence, competition amongst populations, conjugation, antibiotic production, motility, sporulation, and biofilm formation [4].

In the past few years, inhibition of QS has become an intense area of research because of its applications in medicine, industry, and biotechnology. Inhibition of the QS system is considered as a novel strategy for development of antipathogenic agents, especially for combating bacterial infections caused by antibiotic-resistant strains [5]. Therefore, a good strategy to assure food safety and quality could be to regulate food bacterial pathogenesis by inhibiting QS.

Chromobacterium violaceum, a soilborne gram-negative bacterium which resides in the tropical and subtropical areas, synthesizes the violet pigment violacein [6] as a result of QS using its autoinducer N-hexanoylhomoserine lactone. Thus, *C. violaceum* has been used as model bacterium while screening natural products for anti-QS property [7]. Any alteration in the pigment producing ability of *C. violaceum* under the influence of test compounds can easily be quantified photometrically.

Consumers demand safe food with acceptable shelf-life avoiding the use of chemical additives. Therefore, biocontrol approaches are gaining interest, where the use of extracts or pure compounds obtained from plants and fruits are perceived as more "natural" or "green", and offer some specific advantages compared to more conventional treatments. Plant food extracts and phytochemicals have been recognized as effective treatments against food spoilage and pathogen bacteria with the added benefit of antioxidant and other beneficial properties for human health. However, in the last years, the study of anti-QS activity of different plant food extracts and phytochemicals has gained interest [8]. The ability of these natural compounds to interrupt QS systems may serve plants as a defense mechanism to fight against bacterial invasion. One of the keys of success of plant food extracts and phytochemicals could be their similitude to what is considered the ideal QS inhibitor (QSI), which includes being chemically stable, highly effective low-molecular-mass molecules and harmless for human health [9]. The interruption of bacterial QS by plant extracts, although barely studied, is a potential way of controlling microbial pathogenesis [10,11].

Based on this argument, researchers are increasingly investigating herbal products for new therapeutic and antipathogenic agents that might act as non-toxic inhibitors of QS, thus controlling infections without encouraging the appearance of resistant bacterial strains. In the current literature, it is estimated that while 10% of all terrestrial flowering plants have been used by different communities for treating diseases, only approximately 1% have gained recognition and been validated. Thus, phytochemicals and plant extracts may represent the richest available resource of novel therapeutics [12].

Therefore, the present study aims at investigating the effects of three different natural compounds on bacterial QS activity: vanillin, geraniol and pomegranate (*Punica granatum* L.) extract. On previous studies, we have evaluated the antimicrobial effects of these natural agents both *in vitro* [13] and *in vivo* as food biopreservatives on strawberry juice [14]. However, there are few studies where their anti-QS activity is evaluated. Thus, in the present work, the anti-QS activity of vanillin, geraniol and pomegranate extract was tested against *C. violaceum* by measuring the effects of the different natural products in the ability of the bacterium to produce violacein pigment.

Materials and Methods

Bacteria strains, media and culture conditions

C. violaceum wild-type strain American Type Culture Collection (ATCC) 12472 (Malbrán, Buenos Aires, Argentina) was used to determine QS inhibitory and antimicrobial activities. This wild-type strain produces and responds to the cognate autoinducer molecules, AHLs such as C6-AHL and C4-AHL, which makes this strain excellent for screening [15,16]. This bacteriological monitor system generates a phenotypic response by the production of violacein pigment when induced by the presence of AHLs. The bacterium was routinely grown aerobically in Luria-Bertani broth (LB; 1% tryptone, 0.5% yeast extract and 1% NaCl) and incubated at 30 ºC for 48 h.

Natural antimicrobial agents

The natural agents used in this study were two phytochemicals: vanillin and geraniol, both purchased from Sigma Aldrich (St Lois MO, USA); and a fruit extract: (*Punica granatum* L.) pomegranate extract, purchased from PureBulk, USA (35% ellagic acid, 19% gallic acid, 10% punicalagin A, 5% punicalagin B, 2% caffeic acid).

Quantitative QS inhibition assay

Quantification of violacein production: Flask-incubation assays were carried out to quantify the anti-QS activities on violacein production by *C. violaceum* when natural agents were applied. The bacterium was incubated at 30°C for 18 h and inoculated to optical density at 600 nm of 0.1 in erlenmeyer flasks containing LB broth and LB supplemented with bioactive products to obtain different concentrations (0.0625, 0.125, 0.3125, 0.5, 0.625 and 0.75 mg/mL for vanillin; 0.025, 0.05, 0.1, 0.15, 0.20, 0.25 µL/mL for geraniol; and 15, 20, 25, 45, 90 and 180 µg/mL for pomegranate extract). The bioactive products were previously dissolved in dimethylsulfoxide (DMSO). The flasks were incubated at 30 ºC in a shaking incubator for 48 h. The quantification of the violacein production was carried out following the protocol described by Choo et al. [7], where 1-mL culture from each flask was centrifuged at 13,000 rpm for

10 min to precipitate the insoluble violacein. Then, the culture supernatant was discarded, and the pellet was solubilized in 1 mL of DMSO, vortexed until the violacein was extracted, and centrifuged at 13,000 rpm for 10 min to remove cells. Absorbance of each violacein-containing supernatant was measured at 585 nm in a ultraviolet spectrophotometer (Shimadzu Corp., Kyoto, Japan), and the inhibition of violacein production (IVP%) was calculated according to Eq. 1.

$$IVP\% = \frac{OD_{585nmcontrol} - OD_{585nmtreated}}{OD_{585nmcontrol}} 100\% \quad (1)$$

The controls used in this study were LB medium without bioactive, and LB medium + DMSO 1% (v/v). The experiment was carried out three times, and there were three replicates per bioactive agent sample.

Viability assay: The inhibition of violacein production (IVP) of the indicator strain *C. violaceum* could be the result of either (1) quenching of QS signals or (2) inhibition of cell growth [15]. Therefore, antimicrobial activity of bioactive compounds against *C. Violaceum* was evaluated. 10 mL of LB broth were inoculated with 100 µL of an active culture of *C. violaceum*, and the amount of bioactive needed for each selected concentration were added (0.0625, 0.125, 0.3125, 0.5, 0.625 and 0.75 g/mL for vanillin; 0.025, 0.05, 0.1, 0.15, 0.20, 0.25 µL/mL for geraniol; and 15, 20, 25, 45, 90 and 180 µg/mL for pomegranate extract). Inoculated bioactive solutions were mixed followed by incubation at 30 ºC for 48 h. Serial dilutions (1:10) of each inoculated bioactive solution were made in sterile peptonated water (0.1% w/v). Appropriate dilutions were then spread on to LB agar (LB broth supplemented with 1.5% bacteriological agar). Colonies were counted after 48 h incubation at 30ºC. Microbial counts were expressed as log CFU/mL. This assay was performed by triplicate in three separate experimental runs.

Estimation of minimum QS inhibitory concentration

Once the inhibition of violacein production was measured at different concentrations for each natural product, the minimum QS inhibitory concentration (MQSIC) was estimated. According to Alvarez et al. [10] the MQSIC is defined as the effective concentration of bioactive at which 50% of the QS activity is reduced. The following equation was proposed:

$$\log(y) = \beta_o + \beta_1 \log(x) \quad (2)$$

where y is *IVP%,* x is the natural compounds concentration, β_o is the model constant, and β_1 is the linear coefficient.

Statistical analysis

A completely randomized design was used. Three independent runs were performed. Data obtained was analyzed using R v. 2.12.2 [17]. Results reported in this article are mean values accompanied by their standard errors [18]. Analysis of variance ANOVA was performed and Tukey-Kramer comparison test was used to estimate significant differences between treatments ($p < 0.05$).

Estimations of MQSIC's for each antimicrobial agent were developed by linear regression [19] after logarithmic transformation of the violacein production data for different concentrations of each extract, according to the proposed equation (Eq. 2).

Results and Discussion

No significant differences ($p<0.05$) were found between the control with and without DMSO 1% (data not shown). These results mean that the solvent used in this study did not affect the violacein production of *C. violaceum* nor its cell growth. However, to discover quorum sensing inhibitors with more reliability, further control experiments are suggested to avoid false positives. These additional experiments include: (i) verification that the compound does not affect the biosensor phenotype when it is independent of quorum sensing, (ii) assessment of the impact on other phenotypes that are controlled by the quorum sensing system of interest, (iii) transcriptomic analyses, (iv) identification of the molecular target of the compound, (v) sensitive toxicity tests, and (vi) the use of QS inhibition selector systems [20].

Inhibition of QS by vanillin

Figure 1A shows the capacity of vanillin to inhibit violacein production from *C. violaceum* exposed to different concentrations (0 –control sample-, 0.0625, 0.125, 0.3125, 0.5, 0.625 and 0.750 mg/mL). To evaluate whether the inhibition of violacein production (IVP%) was caused by the QS mechanism inhibition or by microbial growth reduction, the *C. violaceum* biomass was also determined. An inverse relationship between pigment production and the applied vanillin concentrations was noticed. Vanillin concentrations from 0.3125 mg/mL showed a significant drop in violacein production. In addition, the cell viability of *C. violaceum* was not affected at concentrations of 0.3125, 0.5, and 0.625 mg/mL; only the highest concentration tested (0.750 mg/mL) significantly reduced the bacterium growth, reducing counts on almost 2.0 log CFU/mL after the incubation period of 48 h.

The interference of QS-dependent processes by natural compounds has been explained by different proposed mechanisms. The process of QS can be disrupted either by (1) reducing the activity of AHL cognate receptor protein and/or AHL synthase; or (2) inhibiting the production of QS signal molecules by different mechanisms such as (a) degradation of the AHL, (b) sequestration of the AHL, and (c) mimicking the signal molecules primarily by using plant food extracts and phytochemicals as analogues of signal molecules [8].

Vanillin, a well-known food flavoring agent, is a phenolic aldehyde (4-hydroxy-3- methoxybenzaldehyde), a phytochemical from vanilla beans (*Vanilla planifolia*). No studies were found where this natural compound was tested as QSI against *C. violaceum*. However, in accordance with our results, vanillin has been recognized as a potential QSI for *Aeromonas hydrophila* biofilms without affecting cell growth in different membranes [21]. Moreover, Ponnusamy et al. [22] also studied the anti-QS activity of vanillin against *Aeromonas hydrophila*, and found that vanillin applied at a concentration of 0.25 mg/mL inhibited 46.3% of the pathogen biofilm formation. These results show that vanillin could be used as a prospective and promising candidate for further exploration as a QSI compound.

Inhibition of QS by geraniol

Similarly, geraniol showed a significant inhibition of violacein production (Figure 1B) compared with control sample. Geraniol was tested at concentrations of 0 (control), 0.025, 0.05, 0.1, 0.15, 0.20, 0.25 µL/mL. From 0.1 µL/mL, every concentration

was effective in reducing violacein production. With respect to the effect of geraniol on the *C. violaceum* viability (Figure 1B), the behavior observed was similar to that found with vanillin. Only the highest concentration tested (0.25 µL/mL) significantly affected the biomass of *C. violaceum*, with approximately 2.0 log reduction compared to control. On the other hand, for geraniol concentrations of 0.1, 0.15, and 0.2 µL/mL, the pigment production inhibition did not affect *C. violaceum* viability, and therefore, could be attributed to a blockage of QS mechanism.

Geraniol is an acyclic monoterpene-alcohol (trans-3,7-Dimethyl-2,6-octadien-1-ol), a phytochemical that occurs in many essential oils like rose, lemon-grass and citronella [13]. Even though, to our knowledge, the anti-QS activity of geraniol has never been studied before, other food phytochemicals have been found to act as QSI not only because of the similarity in their chemical structure to those of QS signals (AHL) but also because of their ability to degrade signal receptors (LuxR/LasR) [16,23]. This could explain the anti-QS activity found for geraniol against *C. violaceum*, although more studies are needed to understand the mechanism of action of geraniol as a QSI.

Inhibition of QS by pomegranate extract

With regards to the effect of pomegranate extract on QS inhibition of *C. violaceum* (Figure 1C), a different trend was observed compared to the ones found for vanillin and geraniol (Figures 1A and 1B). This could be explained by the fact that both vanillin and geraniol are pure compounds, while pomegranate extract includes a variety of substances, which may exert synergism between them and therefore, increase the anti-QS activity at lower concentrations.

Every concentration tested of pomegranate extract (15, 20, 25, 45, 90 and 180 µg/mL) showed significant inhibition on vioalecin production. No differences on IVP% were observed among the concentrations of 90 and 180 µg/mL of pomegranate extract, showing the highest inhibition percentage (more than 70%) of the violacein production. Furthermore, none of the tested concentrations reduced significantly the biomass of *C. violaceum*, which may indicate that the inhibition of the pigment production was the result of quenching of QS signals. These results indicate that the anti-QS activity for pomegranate extract is independent from their effect on cell-growth.

Fruit extracts have also been identified as novel candidates in the search of natural compounds that attenuate bacterial pathogenesis by interfering with QS systems without affecting their viability [11,24]. The disruption of cell-to-cell communication by fruit extracts may reside in the variety of their different phytochemicals that can mimic molecules with the QS signal or accelerate their degradation [25].

In particular, many research studies have focused on the antimicrobial activity of pomegranate (*Punica granatum*) extracts, including leaf, bark, fruit and seed extracts [26]; however, the capacity of pomegranate as QSI has received much less attention. Our results on pomegranate extract's anti-QS activity are in accordance with Truchado, Gil et al. [27] who demonstrated the anti-QS activity of pomegranate extract (20 mg/mL) against *C. violaceum* with more than 70% inhibition of violacein production. These authors also stated that the reduction of AHL production by pomegranate was partially due to the degradation-transformation of AHLs. However, further studies should be performed to confirm the mechanisms by which the tested natural products exhibit their anti-QS activity.

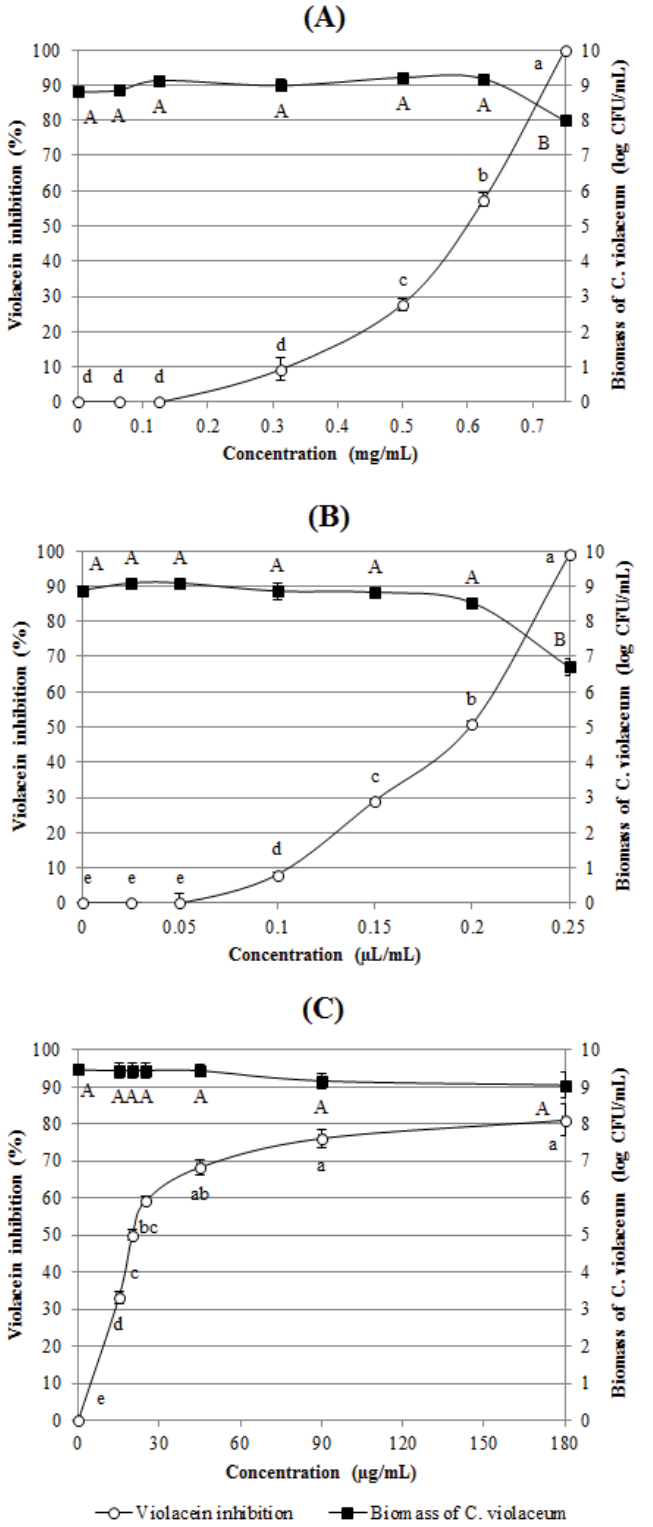

Figure 1: Effect of increasing concentrations of natural products on growth of *C. violaceum* and inhibition of violacein production. **A:** Vanillin; **B:** Geraniol; **C:** Pomegranate Extract. Data is shown as mean value ± standard errors. Different capital letters indicate significant differences in the biomass of *C. violaceum* ($p<0.05$). Different lower case letters indicate significant differences in the inhibition of violacein production ($p<0.05$).

Table 1: Minimum quorum sensing inhibitory concentrations (MQSICs) of natural compounds against *Chromobacterium violaceum* and linear regression parameters.

Natural compound	Adjusted R^2	β_o	β_1	MQSIC
Vanillin	0.959	2.38 ± 0.13	3.08±0.39	0.60 ± 0.02 mg/mL
Geraniol	0.982	3.62 ± 0.16	2.69±0.20	0.19 ± 0.01 µL/mL
Pomegranate Extract	0.848	0.87 ± 0.20	0.59±0.14	25 ± 5 µg/mL

MQSICs and linear regression coefficients (β_o and β_1) are shown as mean value ± standard error.

Determination of MQSICs of natural compounds against *C. violaceum*

Taking into account the results obtained, the minimum QS inhibitory concentration (MQSIC) of the different bioactive extracts was estimated. The MQSIC is designated as the effective concentration of bioactive at which 50% of the QS activity is inhibited.

MQSICs, adjusted R^2 and linear regression coefficients for each antimicrobial agent are shown on Table 1. For every bioactive agent tested, MQSIC was lower than the concentration at which *C. violaceum* biomass significantly decreased from control. Every antimicrobial agent showed low MQSIC, demonstrating their high anti-QS capacity at low concentrations.

Conclusion

The results obtained in this study reflect the potential of vanillin, geraniol and pomegranate extract at low concentrations as promising QS inhibitors, considering that they were able to interrupt intercellular communication inhibiting the violacein production of *C. violaceum* without affecting its growth. Better understanding of the potential of plant extracts and phytochemicals to inhibit QS activity is of great relevance to the field of research aimed at identifying and developing novel anti-QS compounds capable of preventing bacterial infections in humans. Because many important pathogens, as well as spoilage bacteria, use QS to regulate their virulence, strategies designed to interfere with these signaling systems will likely have broad applicability for biological control of disease-causing organisms. Furthermore, the low concentrations needed to exert anti-QS activity, imply less impact of the natural compounds on the sensory quality of the food product, which implies higher consumers' acceptability.

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