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Interrupción de percepción de quórum por fitoquímicos y moléculas sintéticas halogenadas

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QUORUM QUENCHING ACTIVITY OF PHYTOCHEMICALS AND HALOGENATED SINTETIC MOLECULES

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ABSTRACT

In the last decade, the search of new therapeutic alternatives with a mechanism of action different to that of antibiotics has been increasing. The most important is based on the development of antivirulence therapies. To date, the best studied antivirulence target is the quorum sensing inhibition. Quorum sensing or cell to cell communication, is a regulatory mechanism dependent of population density, that promotes multicellular behavior and plays a fundamental role in bacterial virulence expression. The aim of this study was to identify molecules with antivirulence activity (AV-A) from plant and synthetic sources. Through a bioguided assay of hexane and dichloromethane extracts from the bark of *Ceiba pentandra* and *Ceiba aesculifolia*, ten fractions with I-PQ activity against *Chromobacterium violaceum* and *Pseudomonas aeruginosa* were identified, being the last one an opportunistic pathogen classified as critical priority by World Health Organization. On the other hand, new halogenated furanone derivatives were synthesized and evaluated, of which five (C-30, GBr, Dibro A, DEXT 1 and 4) inhibited the production of enzymes, pigments, biofilm and motility against reference and multidrug resistant strains of *P. aeruginosa*. In addition, a mouse abscess model was developed to evaluate the efficacy of these derivatives in a *P. aeruginosa* infection. Two compounds reduced the formation of abscesses, but at the concentration used, it was due to a bactericidal effect. The results obtained indicate that the fractions from barks of the genus *Ceiba* and the halogenated synthetic derivatives are a potential source with A-AV for the treatment of bacterial infections.

Keywords: bacterial communication, virulence factors, antibiotic resistance, pathogenic bacteria, *Ceiba*, halogenated furanones.

INTERRUPCIÓN DE PERCEPCIÓN DE QUÓRUM POR FITOQUÍMICOS Y MOLÉCULAS SINTÉTICAS HALOGENADAS

Naybi Rosario Muñoz-Cázares, D. en C.
Colegio de Postgraduados, 2018

RESUMEN

En la última década, la búsqueda de alternativas terapéuticas con un mecanismo de acción diferente al de los antibióticos ha ido en aumento. La más importante se basa en el desarrollo de terapias antivirulencia, de las cuales la inhibición de la percepción de quórum (I-PQ) ha sido la más investigada. La percepción del quórum es un mecanismo regulador dependiente de la densidad de la población, que favorece el comportamiento multicelular y juega un papel fundamental en la expresión de la virulencia. El objetivo del presente trabajo fue identificar moléculas con actividad antivirulencia (A-AV) de fuentes vegetales y sintéticas. Mediante un ensayo biodirigido de los extractos de hexano y diclorometano de la corteza de *Ceiba pentandra* y *Ceiba aesculifolia*. Se identificaron diez fracciones con actividad I-PQ en *Chromobacterium violaceum* y *Pseudomonas aeruginosa*, esta última un patógeno oportunista clasificado como de prioridad critica por la OMS. Por otra parte, se sintetizó y evaluó la actividad de derivados sintéticos halogenados de furanona, de los cuales cinco (C-30, GBr, A. Dibro, DEXT 1 y 4) inhibieron la producción de enzimas, pigmentos, biopelícula y motilidad en la cepa de referencia y aislados clínicos resistentes a antibióticos de *P. aeruginosa*. También, se evaluó su actividad *in vivo* empleando un modelo de absceso en ratón con *P. aeruginosa*. En el modelo de ratón, dos compuestos redujeron la formación de abscesos. Sin embargo, a la concentración utilizada, se debió a un efecto bactericida. Los resultados obtenidos indican que tanto las fracciones obtenidas de cortezas del género *Ceiba* como los derivados sintéticos halogenados son una fuente potencial con A-AV para el tratamiento de infecciones bacterianas.

Palabras clave: comunicación bacteriana, factores de virulencia, resistencia a antibióticos, bacterias patógenas, *Ceiba*, furanonas halogenadas.

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INTRODUCCIÓN GENERAL

El descubrimiento de compuestos de naturaleza antibiótica ha sido una poderosa herramienta para tratar infecciones de origen bacteriano. Sin embargo, su uso inadecuado por parte de médicos y pacientes, aunado a la capacidad que tienen las bacterias para adaptarse a ambientes adversos, han acelerado la aparición de cepas resistentes a antibióticos (Spellberg *et al*. 2008; Aminov 2010; López-Pueyo *et al*. 2011).

En los últimos años la resistencia bacteriana a ciertos fármacos se ha convertido en un grave peligro para la población humana, por lo que la Organización Mundial de la Salud la ha declarado como un problema de salud pública y ha implementado el "Plan de Acción Mundial sobre la Resistencia a Antimicrobianos", el cual promueve la búsqueda y desarrollo de nuevos fármacos para tratar infecciones bacterianas (Tegos & Hamblin 2013; WHO 2017). Una opción que se ha propuesto recientemente es el desarrollo de "Terapias antivirulencia". Consisten en el empleo de moléculas que tienen como blanco algún sistema o ruta metabólica considerada no esencial para las bacterias patógenas, pero que están estrechamente relacionados con su patogenicidad, como la expresión de factores de virulencia (Escaich, 2010; Tang & Zhang, 2014; Muñoz-Cazares *et al.* 2018).

El emplear moléculas que interfieran con los factores que promueven la colonización e invasión al hospedero, representa un nuevo enfoque terapéutico, ya que contrario a los antibióticos, no actúan eliminando directamente a la bacteria. De esta manera, imponen una menor presión de selección y, en teoría, disminuyen la velocidad con la que aparece resistencia hacia esta clase de antimicrobianos (Clatworthy *et et*. 2007; Escaich 2010; Tang & Zhang 2014).

Dentro de los blancos antivirulencia, uno de los más estudiados es la inhibición de la comunicación bacteriana o percepción de quórum (PQ). La PQ es un mecanismo regulador

dependiente de la densidad de la población, que favorece el comportamiento multicelular y juega un papel fundamental en la expresión de la virulencia bacteriana (Fuqua *et al*. 1994; Waters & Bassler 2005; Rasmussen & Givskov 2006). Se lleva a cabo mediante los Sistemas de Percepción de Quórum (SPQ). Éstos consisten básicamente de dos componentes: el primero es una enzima que secreta moléculas difusibles denominadas autoinductoras (AI) y el segundo es un receptor o regulador de respuesta que reconoce y se activa por medio de estas moléculas. La unión del autoinductor a su receptor desencadena un amplio rango de expresión génica (Williams et al. 2000; Reading & Sperandio 2006; Silva *et al*. 2016).

Cada especie bacteriana puede tener uno a varios SPQ y su activación depende totalmente de la densidad bacteriana (Miller & Bassler 2001). Actualmente, se han reconocido al menos cinco clases de SPQ y se han dividido de acuerdo al tipo de molécula AI que utilizan. Destacan acil-homoserinlactonas, quinolonas, péptidos, ácidos grasos y derivados de la dihidroxipentadiona (Miller & Bassler 2001; Reading & Sperandio 2006; Jimenez *et al*. 2012).

Hasta la fecha se han reportado una gran variedad de moléculas inhibidoras de SPQ (I-SPQ) provenientes de plantas, microrganismos y derivados sintéticos, con diferentes mecanismos de acción. Destacan los siguientes: i) interferir en la unión de las moléculas AI´s y sus receptores, ii) disminuir la concentración del AI en el medio, ya sea por su degradación directa o por la iii) inhibición de la sintasa que lo produce (Vattem *et al*. 2007; Defoirdt *et al*. 2013; García-Contreras 2016).

Los compuestos que destacan por su actividad I-SPQ son los de naturaleza fenólica y específicamente los flavonoides son los principales representantes (Silva *et al*. 2016; Muñoz-Cazares *et al*. 2017). Sin embargo, las furanonas halogenadas producidas por el alga marina *Delisea pulchra* han sido los metabolitos más estudiados por su efectividad frente a diversas

especies patógenas bacterianas y, además, han servido de base para la síntesis de nuevas moléculas inhibidoras (de Nys *et al*.1993; Hentzer *et al*. 2002; Hentzer *et al*. 2003).

Existen varios modelos de sistemas bacterianos que han sido empleados para evaluar la I-PQ, los cuales se basan en determinar la inhibición de fenotipos regulados por uno o varios SPQ. El más común consiste en el empleo de la bacteria *Chromobacterium violaceum*, cuya producción del pigmento violaceína es regulado por un único SPQ, basado en la molécula AI del tipo de las homoserin lactonas (McClean *et al*. 1997; Martinelli *et al*. 2004).

Para el caso de bacterias patógenas se han empleado varias cepas de *Pseudomonas aeruginosa*. Es una bacteria oportunista que causa graves problemas a nivel hospitalario. Está clasificada por la OMS como patógeno de prioridad crítica , debido a su alta capacidad para infectar pacientes inmunosuprimidos, así como de generar resistencia a los antibióticos (Antunes *et al*. 2010; Castillo-Juarez *et al*. 2017; WHO 2017). *P. aeruginosa* regula la expresión de varios factores de virulencia, en donde se destaca la producción de piocianina, pioverdina, rhamnolípidos, y la formación de biopelículas, por medio de tres SPQ basados en AI del tipo homoserin lactonas y quinolonas (Hentzer *et al*. 2003; Adonizio *et al*. 2006; de Kievit 2009;).

Aunque hay una gran variedad de reportes de la actividad I-SPQ de diversas moléculas *in vitro*, los trabajos en los que se demuestre su aplicación y eficacia *in vivo* siguen siendo insuficientes, haciendo necesario el desarrollo de modelos animales de infección adecuados, que permitan determinar más rápidamente su potencial de uso terapéutico frente a infecciones bacterianas (Berube *et al*. 2017; Pletzer *et al*. 2017).

La identificación de moléculas con actividad antivirulencia es un tema de estudio novedoso, que puede ser una estrategia viable para disminuir la aparición de resistencia en donde

uno de los principales blancos de acción es la I-SPQ. De tal forma que el objetivo general de la siguiente investigación fue:

- Identificar moléculas anti-virulencia de fuentes vegetales y derivados sintéticos halogenados

Los objetivos particulares fueron:

- Evaluación de la actividad I-SPQ de metabolitos provenientes de dos cortezas del género *Ceiba*, usadas en la medicina tradicional mexicana, en la producción de violaceina en *Chromobacterium violaceum* y factores de virulencia en *Pseudomonas aeruginosa*.
- Evaluar la I-SPQ de nuevos derivados sintéticos halogenados en cepas resistentes a antibióticos de *P. aeruginosa*.
- Desarrollo de un modelo de absceso en ratón para evaluar la I-SPQ de moléculas sintéticas halogenadas en la infección por *P. aeruginosa.*

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CHAPTER I. PHYTOCHEMICAL SCREENING AND ANTI-VIRULENCE PROPERTIES OF *Ceiba pentandra* AND *Ceiba aesculifolia* BARK EXTRACTS AND FRACTIONS

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Phytochemical screening and anti-virulence properties of *Ceiba pentandra* and *Ceiba aesculifolia* bark extracts and fractions

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1.1 Abstract

Background: The emergence of resistant bacteria to antibiotics is a problem that need to be carried with new therapeutic strategies. In this sense, the development of anti-virulence therapies has been increasing for the treatment of bacterial infections, focusing mainly on the inhibition of bacterial quorum sensing systems (QSS-I). Plant sources have been very useful to obtain metabolites with this inhibitory activity and species in the Mexican flora represent an important repertory for the obtention of metabolites with QSS-I

Species studied: *Ceiba pentandra* and *Ceiba aesculifolia*.

Study site and years of study: The *Ceiba* barks were collected in the municipalities of Tierra Blanca and Acatlan, states of Veracruz and Oaxaca respectively in august of 2013.

Methods: we determined the effect of hexane and dichloromethane extracts of *C. aesculifolia* and *C. pentandra* over phenotypes-QSS regulated of *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. Extracts were fractionated and the main metabolites were identified. A histological study of the bark was made to define distinctive features to facilitate the identification of the samples.

Results: QSS-I activity was found in the extracts of both species of *Ceiba*. Four fractions were identified with the ability to attenuate the production of virulence factors in *P. aeruginosa*. These are rich in terpene and sterol compounds. Histological analysis revealed some differences that facilitate the identification of the two species.

Conclusions: It was confirmed that traditional use of these plants to fight infections through a mechanism complementary to antibiotic effect. Similarly, they are important sources for obtaining QSS-I metabolites.

Key words: antibiotic resistance, bacterial communication, Mexican plant, pochote, quorum sensing systems.

1.2 Introduction

An emerging problem associated with the indiscriminate use of antibiotics is the selection of bacteria with highest resistant levels against antibiotics (Fischbach & Walsh 2009). The therapeutic options are becoming limited which represent a serious problem that urgently require to be addressed (Roy *et al*. 2011). Inside the new anti-virulence strategies to combat resistant

bacteria, one of the most proposed and studied is the inhibition of bacterial quorum sensing systems (Muñoz-Cazares *et al*. 2017).

Bacterial communication or quorum sensing is a regulatory mechanism dependent of the population density, that favours the multicellular behaviour in the bacteria. Is carried out by the quorum sensing systems (QSS) which consist in the production, diffusion, detection and responses to chemical signalling molecules known as autoinducers that play a fundamental role in the expression of some phenotypes like pigments, bioluminescence, siderophores and in the case of bacterial pathogens the production of virulence factors and biofilm formation (de Kievit 2009; Stauff & Bassler 2011; Koh *et al*. 2013)

The quorum sensing system inhibition (QSS-I) may be a useful alternative to antibiotics since quorum sensing controlled phenotypes that are considered as new target for antimicrobial chemotherapy (Zhang & Dong 2004; Adonizio *et al*. 2006). The QSS-I unlike antibiotics repress the expression of virulence factors and biofilms without affecting the bacterial viability (Rasmussen & Givskov 2006; Fischbach & Walsh 2009) . As a result, it is postulated that the bacterium does not develop resistance and the immune system eliminates the infection (Roy *et al*. 2011).

New investigations have focused to discover agents derived from synthetics and natural products to handle the bacterial pathogenesis by means of QSS-I (Pan & Ren 2009). In Mexico around 4000-5000 species of plants have medicinal properties which are frequently used for treat several medical affections (Espinosa *et al*. 2008; Valdivia-Correa *et al*. 2016); in this sense the trees of "pochote" or "pochotl", a term used in the traditional nomenclature to refer several species of the genus *Ceiba* spread in different regions of Mexico (Gibbs & Semir 2003), are widely used for therapeutic applications. Specially in the central part of Mexico, the barks of *Ceiba pentandra*

and *Ceiba aesculifolia* are used to cure kidney disorders and skin infections, as well as to decrease blood sugar levels (Canales *et al*. 2005).

Also there are several reports of the antibacterial activity of the bark, seeds and fruit of *C. pentandra*, in despite of being native to Central America, it has been introduced to various regions of the world (SEMARNAT 2013), in contrast *C. aesculifolia*, is native from the Mexican tropical dry forest and there are less reports of this bactericidal activity (Canales *et al*. 2005; Niembro & Sánchez 2010). Furthermore the biological activities reported for both species are similar (Ladeji *et al*. 2003; Canales *et al*. 2005) and probably in local markets not are differences in the commercial distribution of the barks, making important have a method for the identification between species.

In this study, we evaluated the properties of the *C. aesculifolia* and *C. pentandra* barks to inhibit quorum sensing systems in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*, in order to identify a source of anti-virulence metabolites.

1.3 Materials and methods

1.3.1. Plant material

Stem bark of *Ceiba aesculifolia* (Kunth) Britten & Baker f., was collected in the municipality of Tierra Blanca, state of Veracruz, Mexico: at coordinates 18° 34.189’ N and 096°22.690’W. For *Ceiba pentandra* (L.) Gaertn., samples were obtained in the municipality of Acatlán, state of Oaxaca, Mexico; at coordinates 18°32.677’N and 096°36.336’W. The botanical identification was carried out by Dr. Antonio Guízar Nolasco (Dicifo/UACh), two vouchers specimens were deposited at the Herbarium of the Universidad Autónoma Chapingo (CHAP), with register number: *C.pentandra* 66,486 and *C. aesculifolia* 66, 487.

1.3.2. Extracts preparation and fractionation.

The air-dried and powdered barks of *Ceiba* (CpB and CaB) (1.0 kg) were sequentially extracted with hexane (Hex), dichloromethane (D), and methanol (MeOH) (J.T. Baker®). The solvent was evaporated under low pressure.

The CpB and CaB D and Hex extracts were subjected to a vacuum column chromatography (CC) using sílica gel 60 (70-230 mesh, Merck®) and eluted with different mixtures of Hex-Ethyl acetate (EtOAc) and EtOAc:MeOH (J.T. Baker®), resulting in eight fractions of CaBHex, 12 fractions from CaBD, CpBHex six fractions and 12 final fractions in CpBD. The obtained fractions were concentrated and analyzed by thin layer chromatography (TLC). Analyses of TLC were performed according to conventional techniques, using plates of 0.25 mm thick support aluminum (60 F254 Merck®). The plates were visualized under UV light and subsequently revealed with 2% vanillin-10 % H_2SO_4 in ethanol, followed by heating (110 °C) for the full display of the compounds.

1.3.3. Phytochemical screening

Active fractions were examined for the presence of common classes of plant secondary metabolites by TLC with various reagents to detect alkaloids, flavonoids, phenols, tannins, terpenes, triterpenes and steroids, following the methods described by Harborne (Harborne 1984).

1.3.4. Anti-quorum sensing activity in Chromobacterium violaceum

Two biomonitor strains were used, ATCC553 a wild-type strain which synthesize violacein a quorum sensing controlled purple pigment. The production of violacein is regulated by the C4 and C6 homoserine lactones autoinducer molecules (AHL). The other one, CV026 is a mini Tn5 mutant indicator strain derived from wild type CV31532 strain and is unable to synthesize its own AHL but retains the ability to respond against exogenous AHL.

The effect of extracts and fractions on the quorum sensing controlled production of violacein was determined using the wild-type ATCC553 strain, while the potential toxic effects on growth was monitored using non-pigmented CV026 strain. For the inhibition of violacein production and bacterial growth the multi-well system assay was used. Polystyrene 96-well microtiter plates were used and 200 µL of the cultures adjusted to an optical density at 600 nm = 0.05 (10^5 CFU/mL^{-1}) (Multiskan Spectrum) seeded in each well. Extracts and fractions were dissolved in dimethyl sulfoxide (DMSO) and 5 µL added to the cultures to final concentrations of 100 and 200 µg/mL. For all the assays at least three independent cultures were included.

They were incubated at 25°C; 120 rpm for 48 h. DMSO and LB medium was used as negative control and anacardic acid mixture (AA) 100 µg/mL as positive control (Castillo-Juárez *et al*, 2013). The violacein obtained after drying the culture media was resuspended in 200 µL of DMSO and the absorbance was measured at 590 nm. In order to calculate the percentage of inhibition, the absorbance of the negative controls was taken as 100 % of violacein production. The growth was determined by the absorbance of the cultures at 600 nm and the inhibition percentage was calculated by subtracting the absorbance of the extracts and fractions, as well as considering the value LB medium controls as 100% of growth.

1.3.5. Anti-quorum sensing activity in Pseudomonas aeruginosa

To test the expression of virulence factors a PA14 wild-type was used. For all experiments were cultured in LB medium at 37ºC in aerobic conditions with shaking at 200 rpm for 20 h. The extracts and fractions were dissolved in DMSO and 5-10 µL added to 5 mL of the cultures adjusted to an optical density at 600 nm=~ 0.05 (UV-1800, Shimadzu), with a final concentrations of 128 to 500 µg/mL. DMSO was used as negative control and the production of all the virulence factors was

normalized by growth (absorbance 600 nm). For all the assays at least three independent cultures were included.

Pyocyanin production was determined spectrophotometrically after the extraction with chloroform from the cultures and a further extraction with 0.2 N HCl. The pyocyanin concentration was estimated from the peak to an optical density at 520 nm with a millimolar extinction coefficient of 2.46 mM^{-1} cm^{-1} (O'Malley *et al*. 2004). The pyoverdine present in the supernatants was assayed spectrophotometrically by absorbance at 407 nm, diluting the supernatants 1:10 in distilled H_2O (Ren *et al*. 2005a).

The alkaline protease activity was detected spectrophotometrically by the Hide-remazol blue assay, the absorbance was measured at 595 nm (Howe & Iglewski 1984). The quantitation of the elastolytic activity in the supernatants was determined by the elastin- congo-red (ECR) SIGMA assay, according to a previously procedure reported (Maeda *et al*. 2012).

1.3.5. Histology methods

Segments of the inner and outer bark (3 x 2 cm) of four individuals of *C. aesculifolia* and *C. pentandra* were obtained at a height of 1.30 m from the main stem. Subsequently incorporated into a solution of ethyl alcohol-glycerin-water (GAA, 1:2:3) for a period of 30 days to soften them.

For microtechnical procedure, cuts were made from 20-30 µm thick in the transversal, tangential and radial view, using a sliding microtome. In the case of the tangential plane, serial cuts were made from the bark to the vascular cambium. Sections were stained with safranin-fast green to be mounted in synthetic resin.

The bark anatomical description of *Ceiba*, was made following the terminology of Trockenbrodt (Trockenbrodt 1990). Images were obtained using the analyzer elements NIS-BR 2.33 (Nikon corporation 1991-2006). The general drawings were prepared using a camera Lucida to 1X on a Nikon microscope Labophot-2.

Statistical analyses. The results are presented as the average and standard deviation of at least three independent experiments. The Student's t test for non-paired samples was used for statistical analysis. These analyses were done in IBM-SPSS 22v software.

1.4 Results

1.4.1. Activity of the extracts in the production of QSS-regulated phenotype of C. violaceum and P. aeruginosa.

QSS-I was recorded mainly in the CaBD, as well as in the CpBHex and CpBD, inhibiting the production of violacein up to 60% and showing non significative effects on the viability of CV026 strain (Figure 1). There was a reduction on the production of pigment with methanol extracts of the two species of *Ceiba*, and the CaBHex, however, also significant effects on growth inhibition of CV026 strain were found (Figure 1.1).

In these assays, AA was employed as a positive control, since in a previous study it was reported that these molecules inhibit the production of violacein (Castillo-Juárez et al., 2013). In *P. aeruginosa* significant inhibitory activities were only found in CaBHex and CaBD at the highest dose (Table 1.1).

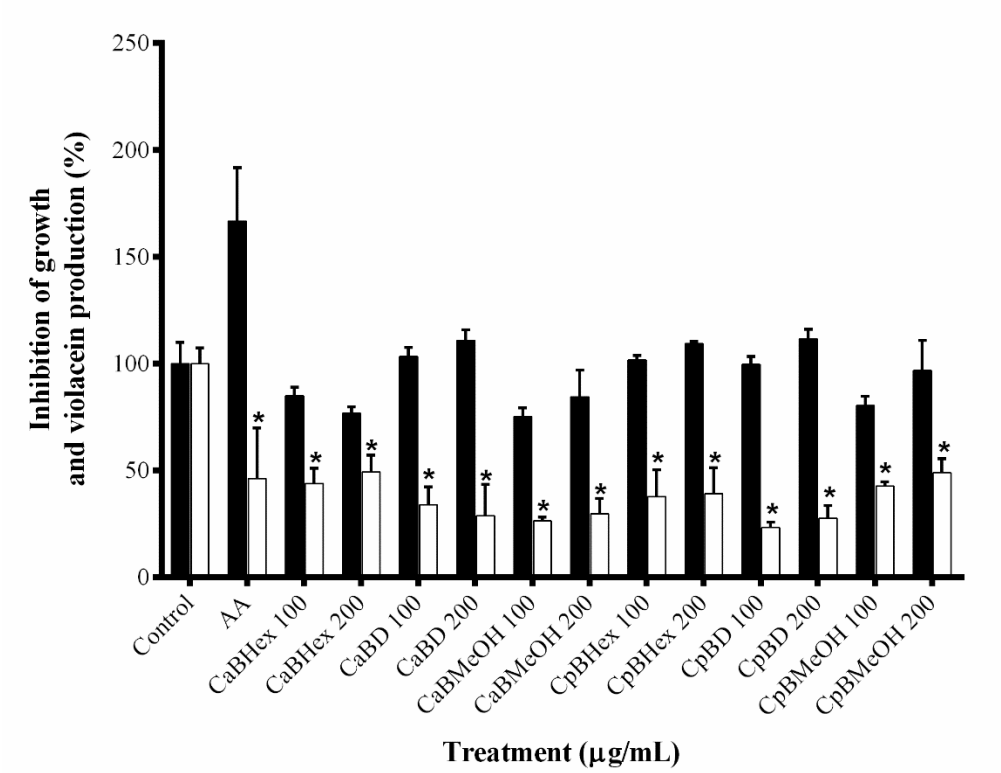

Figure 1.1. Inhibition of violacein production by *Ceiba aesculifolia* and *Ceiba pentandra* bark extracts. Black bars represent bacterial growth (mutant CV026) and white bars represent violacein production (CV12472 WT). AA = anacardic acids mixture. CaB=*Ceiba aesculifolia* bark and CpB=*Ceiba pentandra* bark. Extracts: HEX = hexane, D= dichloromethane, MET = methanol. *P<0.05 versus control group.

Table 1. 1. Effect of the extracts of *Ceiba aesculifolia* and *Ceiba pentandra* in the production of virulence factors on *Pseudomonas aeruginosa*.

	Percent inhibition of virulence factors (384 µg/mL)		
Specie/extract	Pyocyanin	Pyoverdin	Elastolytic activity
C. pentandra hexane	2.5	nd	16.5
C. aesculifolia hexane	9.5	16	26.5*
C. aesculifolia dichloromethane	30.5 *	30.5 *	32*

**P* (< 0.05). nd = not determined

1.4.2. Effect of the fractions on the production of QSS-controlled virulence factors of C. violaceum and P. aeruginosa.

The fractions showed a different activity stimulating or inhibiting virulence, being F1CaB/Hex, F6CaB/D, F1CpB/Hex and F3CpB/D the most active fractions against the pyocyanin and alkaline protease activity in *P. aeruginosa*, interestingly whereas this fractions inhibit virulence factors in this bacteria, in *C. violaceum* stimulates the production of violacein or showed a discrete inhibitory effect. The yield of the fractions and system of elution are shown in Table 1.2.

1.4.3. Dose-response QSS-I effect of fractions on the alkaline protease activity of P. aeruginosa

The dose-response effect of active fractions F1CaBHex, F6CaD, CpBHex and F3CpD over alkaline protease activity and growth of *P. aeruginosa* was analyzed and were only observed for F1CpBHex and F1CaBHex fractions (Figure 1.2). It should be noted that the fractions at higher doses of 250 µg/mL, presented solubility problems in the cultures, a phenomenon that may be responsible for not be clear the dose-response of some fractions.

1.4.4. Principal groups of metabolites present in the active fractions

The active fractions were screened for the presence of common classes of plant secondary metabolites (Table 1.3). The four fractions analyzed (F1CaHex, F6CaD, F1CpHex and F3CpD), are composed mainly of terpene-type metabolites. In F1CaHex, F6CaD and F1CpHex the triterpene and steroidal type of compounds were detected, whereas in F6CaD and F1CpHex, the presence of a major compound, which gave positive the test of flavonoids was found.

Table 1. 2. Effect of the fractions of *Ceiba aesculifolia* and *Ceiba pentandra* on the violacein production in *Chromobacterium violaceum* and two virulence factors of *Pseudomonas aeruginosa*

fraction/specie/extract/proportion/ yield	***C. violaceum* (200 µg/mL)**		***P. aeruginosa* (500 µg/mL)**	
	%I violacein	I% growth	%I virulence factors	
			pyocyanin	alkaline protease activity
F1CaBHex/*C. aesculifolia*/Hex/9H:1A/485	+3	+3	**37.4***	**65.9***
F2CaBHex/*C. aesculifolia*/Hex/8H:2A/352	42*	-7	+93.9	+92.3
F3 CaBHex/*C. aesculifolia*/Hex/6H:4A/282	45*	-3	+19.8	+2.21
F4 CaBHex/*C. aesculifolia*/Hex/3H:7A/129	60*	-16	+28.36	+53.28
F5 CaBHex/*C. aesculifolia*/Hex/9A:1M/8.6	69*	+15	+69.82	+56.42
F6CaBD/*C. aesculifolia*/D/9H:1A/115	+40*	-15	**40.6***	**59.9***
F7 CaD/*C. aesculifolia*/D/100A/54	22*	+23	+70	+125
F1CpBHex/*C. pentandra*/Hex/8H:2A/520	**34***	+5	**40.7***	**80.4***
F2CpHeB/*C. pentandra*/Hex/6H:4A/310	46	+15	+14.5	+53.4
F3CpBD/*C. pentandra*/D/8A:2M/370	**24***	+20	**31.1***	**65.5***

%I= percent inhibition. Hex= hexane extract. D = dichloromethane extract. H = hexane. A = ethyl acetate. M = methanol. Yield = mg for every Kg. **P* (< 0.05).

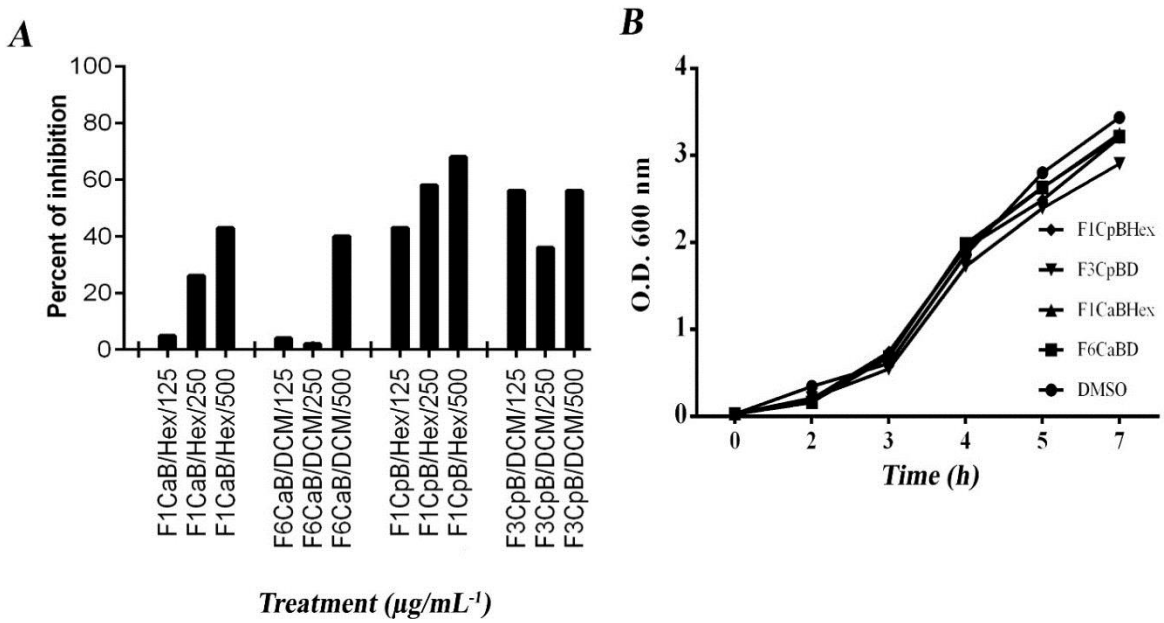

Figure 1. 2. Dose-response effects of the fractions of *Ceiba aesculifolia* and *Ceiba pentandra* in the production of QSS-controlled virulence factors. A) Alkaline protease activity. B) Growth. In the graphs: DMSO = dimethylsulfoxide. *C. aesculifolia* fractions: F1CaHe and F6CaD. *C. pentandra* fractions: F1CpHe and F3CpD. Data show the average of at least three independent experiments.

1.4.4. Anatomical differences between the barks of C. aesculifolia and C. pentandra

In cross section, there are clear differences between *Ceiba* barks analyzed (Figure 1.3). *C. pentandra* (Figure 1.3 E-H) shows narrow fiber bands in the non-collapsed phloem (Figure 1.3H) and short rays dilate near the vascular cambium (Figure 1.3E). Druses in *C. aesculifolia* are numerous (1.3D) while prismatic crystals are numerous in *C. pentandra* (Figure 1.3H).

The sclereids groups in the dilated rays are large and tangentially elongate in the collapsed phloem of *C. pentandra* (Figure 1.3G); unlike in *C. aesculifolia* they are irregularly rounded, smaller and close to periderm (Figure 1.3C). In *C. aesculifolia* the prickles are stratified, 2-3 layers of cells with clear lumens and thin walls are alternating with numerous layers of cells with darker lumens and thicker walls (Figure 1.3B); this is repeated (1.3A) up to more than five times in some

prickles. In case of *C. pentandra*, the prickles are smaller and form a homogeneous tissue composed of numerous cell layers of phellem elongated radially (Figure 1.3E, F). The table 1.4 summarizes some distinctive features of both species

Table 1. 3. Results of phytochemical screening of the active fractions of *Ceiba aesculifolia* and *Ceiba pentandra*.

Metabolites	**Test/reagent**	**Result**			
		F1CaBHe	**F6CaBD**	**F1CpBHe**	**F3CpBD**
Terpenoids	2% Vanillin/ 10% H_2SO_4 ethanol	positive	positive	positive	negative
Flavonoids	1%NP /5%PEG	negative	positive	positive	negative
Alkaloids	Dragendorff	negative	negative	negative	negative
Steroids and triterpenoids	Liebermann-Buchard	positive	positive	positive	positive
Tannins	$FeCl_3$/Folin-Ciocalteu	negative	negative	negative	negative
Phenols	$FeCl_3$/Folin-Ciocalteu	negative	negative	negative	negative

Table 1. 4. Anatomical characteristics of the barks of *Ceiba aesculifolia* and *Ceiba pentandra*.

Characteristics	*C. aesculifolia*	*C. pentandra*
Rays	Slightly dilated Toward the outer bark	Strongly dilate near the vascular cambium
Form of sclereids groups	Small and rounded	Large and tangentially elongate
Position of sclereid cells	Present toward periderm; Scarce	In phloematic ray and larger ones, towards the outer bark Numerous
Fiber groups in non collapsed phloem	Not evident	Evident
Druses	Abundant	Scarce and regular
Prismatic crystals	Regular	Abundant
Prickles	Stratified	Homogeneous

CaB= *C. aesculifolia* bark CpB= *C. pentandra* bark He=Hexane D= Dichloromethane

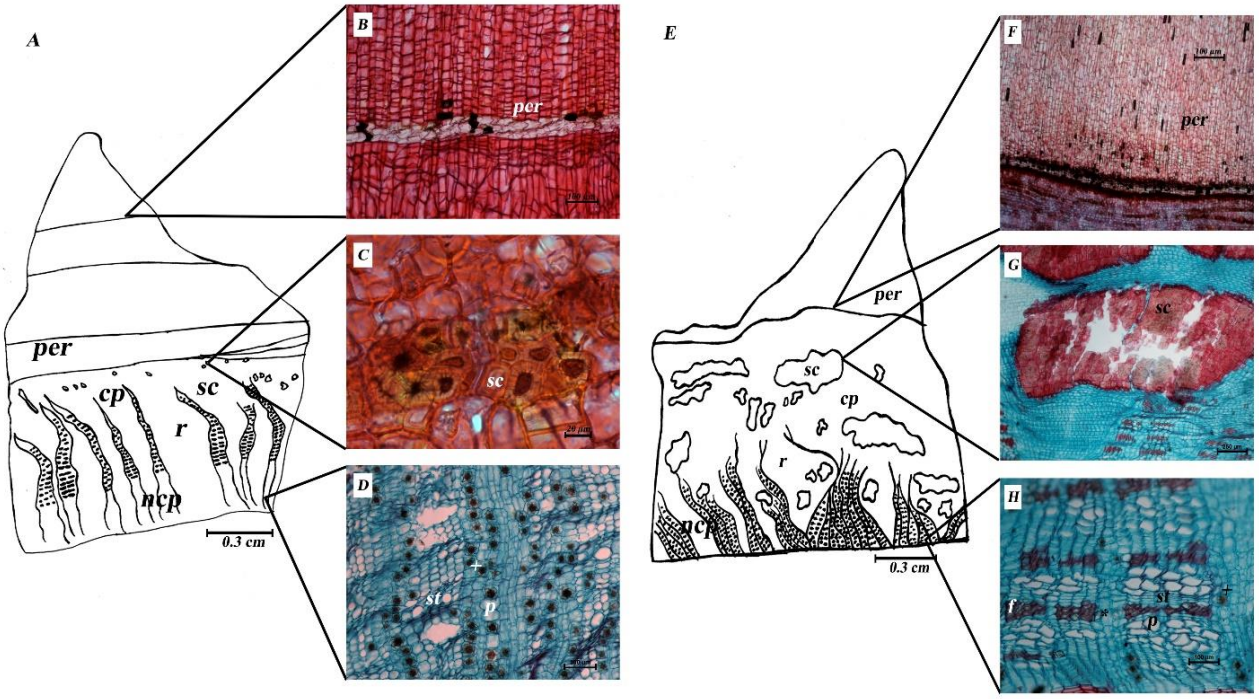

Figure 1. 3. Sections of the barks of *Ceiba*: A-D) Transverse sections of *Ceiba aesculifolia*: B) periderm, C) form and position of sclereids group, D) non-collapse phloem. E-H) Transverse sections of *Ceiba pentandra*: F) periderm, G) form and position of stone cells, B2) non-collapse phloem. Abbreviations: cp = collapse phloem; f = fibers; ncp= non-collapse phloem; p = parenchyma; per = periderm; r = ray; st = sieve tube; sc = stone cells; * = druses; + = prismatic crystal.

1.5 Discussion

The bacteria-plant interaction, is a phenomenon that has developed over thousands of years, during which they have perfected evolutionary strategies, which involve the production of metabolites with ability to regulate (positively or negatively) bacterial QSS (Nazzaro *et al* 2013).

The *C. aesculifolia* and *C. pentandra* barks showed QSS-I activity, indicating the presence of active metabolites. Differences in the activity recorded in the two biological systems used may be related to the complexity of the QSS of bacterial species.

C. violaceum is an aquatic bacterium that can infect humans and cause abscesses and bacteremia (Stauff & Bassler 2011), the wild type strain and biosensor mutants of this bacterium are widely used in the study of QSI by naturals products (Steindler & Venturi 2007), since the well-studied trait controlled by quorum sensing is the production of the hallmark purple pigment violacein. The bacteria *P. aeruginosa* is an opportunistic pathogen that represent a major health problem worldwide being responsible of 10% of nosocomial infections (Antunes *et al*. 2016); and is classified as a pathogen of critical priority by the World Health Organization (WHO 2017). For this bacterium to date, three QS interrelated systems were reported that regulates the production of virulence factors such as pyocyanin, pyoverdine, alkaline protease and elastolytic activity among others (Christensen *et al*. 2007; Gellatly & Hancock 2013).

Global QSS-I effect presented by the active extracts, changed when the fractions were analyzed, because it was found that several stimulated or inhibit virulence factors. Also, some had a slight antibiotic effect over the strains. These results indicate that the diversity of metabolites with regulatory activity present in the two *Ceiba* species is complex. Regulatory behavior over QSS (positive or negative) by different fractions was displayed, like changes in relation to the increase of the concentration, caused by the fractionation as well as for the selectivity of the molecules over each QSS.

In bioassays, doses above 250 µg/mL, presented solubility problems, it is possible that the lack of dose-response effect of some fractions on pyocyanin and alkaline protease activity is due to this phenomenon.

The search and identification of QSI molecules within extracts, is complex, due to the diversity of metabolites ability to regulate differently the QSS. Similarly, it is necessary to

investigate the effect of separate molecules, to define their selectivity and antagonistic effects with other bacterial QSS as well as their action mechanisms.

However, the analysis for the identification of the major groups of metabolites in the fractions active against *P. aeruginosa* system revealed that were mainly composed of terpenes, triterpenes and sterols. This result is important, because there are few reports of this type of metabolites as QSS-I.

In literature, the anti-biofilm and anti-virulence activity of pentacyclic triterpenes derivatives (oleanane, corosolic, asiatic and ursane) against *E. coli*, *S. aureus*, *P. aeruginosa* and *V. harveyi* were reported (Eldrige 2005; Ren *et al*. 2005b; Hu *et al*. 2006; Garo *et al*. 2007; Gilabert *et al*. 2015). Also sterols from *Dalbergia* species showed inhibitory activity against virulence factors of *P. aeruginosa* (Rasamiravaka *et al*. 2013). The active fractions can be an important source for the search of new terpenes-sterols-type metabolites, with potential to expands the repertoire of QSS-I molecules.

The results also showed the presence of antibiotic molecules within the extracts or fractions may mask the QSS-I activity, the major example were the extracts of *C. aesculifolia* which reduced the violacein production, while affecting the viability. Considering only the global activity of the extract, the presence of antibiotic molecules can be complementary to that of QSS molecules to provide more potent anti-virulence effect. That was demonstrated in *P. aeruginosa* when the asiatic and corosolic acid increased the susceptibility to tobramycin in this bacterium (Garo *et al*. 2007).

Other reports also showed that others QSS-I molecules from natural sources, can potentiate the effect of antibiotics against pathogenic bacteria (Rasmussen & Givskov 2006; Pan & Ren 2009). This activity can facilitate the therapy with antibiotics and hence increasing its

effectiveness, favoring the use of low doses and avoiding the indiscriminate use of broad-spectrum antibiotics (Bjarnsholt & Givskov 2007).

Three species of *Ceiba* are recognized in the central part of Mexico, but when no flowers or fruits are collected, diversity and varieties of "pochotes" may lead to misidentification of the new sources described. In this sense, the anatomical characteristics of bark may be helpful in species identification (SEMARNAT 2013).

We report various bark features that anatomically distinguish one species from another. Rays dilation close to vascular cambium, fibers evident in non-collapse phloem, as well as the position, size and form of sclereids groups are the most noticeable. These distinctions may serve as quality controls of these plants. In Mexico, pharmacological researches have been supported by anatomical studies of bark to help distinguish species of medicinal importance (Rivera-Arce *et al*. 2007; Rosas-Acevedo *et al*. 2011).

In this work we identified the QSS-I activity of extracts and fractions of the barks of *C. aesculifolia* and *C. pentandra*. Also, active fractions were identified which contain predominantly terpenes and sterols, poorly studied QSS-I molecules in contrast with other groups of metabolites. These results provide evidence to validate the traditional use give to the barks in central areas of Mexico, therefore *Ceiba* species can be considered a new source for obtaining QSS-I active metabolites.

1.6 Acknowledgments

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CHAPTER II. NEW SYNTHETIC HALOGENATED FURANONES AS ANTIVIRULENCE INHIBITORS AGAINST *Pseudomonas aeruginosa*

New synthetic halogenated furanones as antivirulence inhibitors against *Pseudomonas aeruginosa*

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2.1. Abstract

Antivirulence therapy is one of the most promising strategies to combat multidrug-resistant bacteria, in which the inhibition of quorum sensing is an important target, as it regulates virulence in several pathogenic bacteria. To date natural or derivative furanones have demonstrated their ability to attenuate bacterial virulence. Exploring new synthetic halogenated furanone derivatives with structural changes to potentiate their inhibitory effects is an attractive goal. In this study we demonstrate the quorum quenching activity of new HFDs against antibiotic-resistant strains of *P. aeruginosa*. All the HFDs markedly inhibited biofilm formation and swarming motility in all isolates tested, whereas the inhibition of alkaline protease or pyocyanin were variable. Furthermore, in a mice infection model, two compounds reduced the formation of abscess, but at

concentration used the result obtained was due to a bactericidal effect. Despite the inhibitory effects shown by the compounds *in vitro*, different doses and toxicity in living models need to be assessed to confirm that the results are related to quorum quenching activity.

Key words: antibiotic resistance, virulence factors, quorum quenching, mouse abscess model, furanone C-30

2.2. Introduction

The constant appearance of multidrug-resistant bacteria represents one of the major challenges for human health. Although the discovery of antibiotics revolutionized the field of medicine and saved millions of lives, their indiscriminate use potentiated the capacity of acquisition and resistance spread among bacterial species (Crofts *et al*. 2017; Fair & Tor 2014).

To combat this worldwide problem the World Health Organization (WHO) in 2015 launched antimicrobial strategies and action plans, encouraging the research, discovery and development of new antimicrobials (Piddock 2017; WHO 2017). These action plans are primarily focused on bacteria classified as *critical priority* like *Pseudomonas aeruginosa*, an opportunistic pathogen responsible of 10% of nosocomial infections including pneumonia, bacteremia, urinary tract infections, ocular diseases and a spectrum of infections that involve abscess formation (Antunes *et al*. 2010; Berube *et al*. 2017; Castillo-Juarez *et al*. 2015).

The antivirulence therapy is a novel strategy to combat bacterial resistance, in which the principal objective is inhibit the virulence factors that pathogens require to cause damage without affecting the viability of the bacteria (Mühlen & Dersch 2015; Ruer *et al*. 2015; Defoirdt 2016; Muñoz-Cazares *et al*. 2018). Among antivirulence targets, the inhibition of quorum sensing (QS) (bacterial cell-to-cell communication) is one of the most studied. It has been shown to be a regulatory mechanism dependent of population density, that initiates the multicellular behavior

and plays a fundamental role in virulence expression (Fuqua *et al*. 1994; Waters & Bassler 2005; Rasmussen & Givskov 2006).

The QS is carried out by quorum sensing systems (QSS) which consist of an enzyme that secretes small diffusible signals called autoinducers and a receptor or response regulator. The binding of the autoinducer to their receptor trigger a wide range of gene expression (Williams *et al*. 2000; Zhang & Dong 2004; Silva *et al*. 2016). *P. aeruginosa* has three interrelated systems of QS that regulate the production of virulence factors such as pyocyanin, pyoverdine, alkaline protease and elastolytic activity; and their persistence also be associated with biofilm formation (Steindler & Venturi 2007; Jimenez *et al*. 2012; Lee & Zhang 2015).

The inhibition of QSS on *P. aeruginosa* by diverse compounds called quorum quenchers (QQ) from natural or synthetic sources is widely documented (Adonizio *et al*. 2006; Rasmussen & Givskov 2006; Janssens *et al*. 2008; Tang & Zhang 2014; Moore *et al*. 2015). One of the best studied and most effective QQ are the synthetic brominated furanones C-30 and C-56, which are derivatives of halogenated furanones (HFs) produced by the marine alga *Delisea pulchra* (de Nys *et al*. 1993; Hentzer *et al.* 2002; Hentzer *et al*. 2003; Wu *et al*. 2004; Maeda *et al*. 2012).

An important feature to consider is the acquisition of resistance of *P. aeruginosa* against QQ, found commonly in clinical isolates. This means that it is advisable to use combination therapies, exploiting the inhibition of QSS and the use of other agents with different virulence targets (Maeda *et al*. 2012; Kalia *et al*. 2014; García-Contreras *et al*. 2015; García-Contreras 2016).

Chemical synthesis is an effective approach in design and development of new drugs, and in the case of HFs their capacity for structural change with the conservation of the 2(5H)-furanone moiety, may enhance their capacity to be antivirulence agents, enhancing their inhibitory effects without affecting bacterial growth (Janssens *et al.* 2008; Shetye *et al*. 2013; Abdel-Rahman *et al*.

2017). Following this approach our research group synthesized seven HFs derivatives structurally related with natural furanones with systematic changes in their structures (Figure 1).

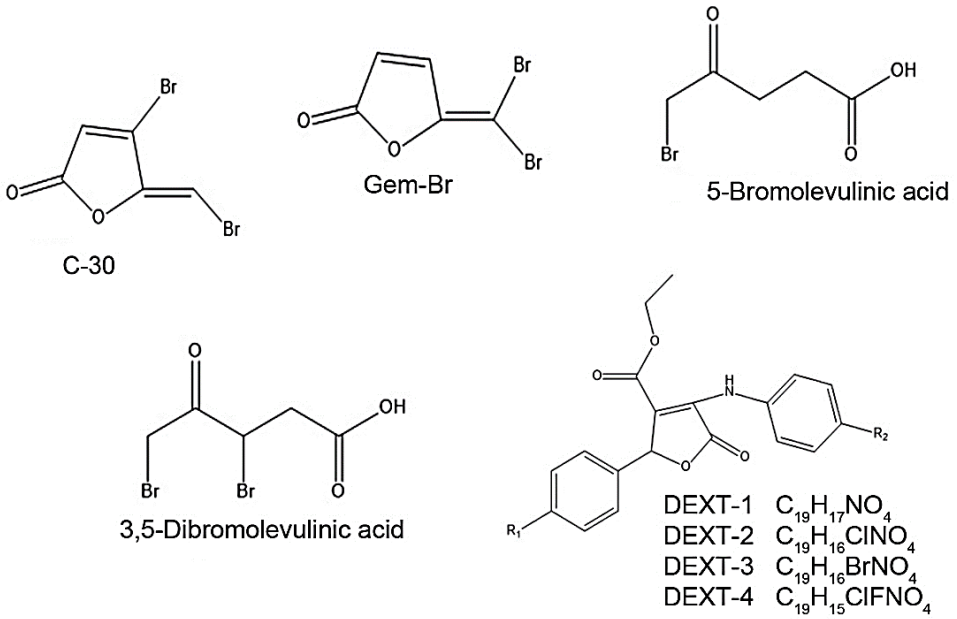

Figure 2. 1. Chemical structures of the C-30 and new halogenated furanone derivatives used in this study.

In this study, we evaluated the QQ activity of new synthetic halogenated furanones derivatives against *P. aeruginosa* clinical isolates. In addition, a mouse abscess model was used to demonstrate the efficacy of these derivatives in *P. aeruginosa* infection.

2.3. Material and methods

Synthetic halogenated furanones derivatives

Furanone C-30, halogenated derivatives GemBr (GBr), 5-Bromolevulinic acid (Bro. A), 3,5-Dibromoevulinic acid (DBro. A) and Dext-1 to 4 were chemically synthesized by Dr. Mariano Martínez Vázquez and MSc Victor Castro Torres, Laboratory of Natural Products, Instituto de Química, Universidad Nacional Autónoma de México, México (publication in progress). C-30 and

furanone derivatives were dissolved in dimethyl sulfoxide (DMSO) and added to the cultures to final concentrations of 10, 50 and 100 µM (Figure 1).

2.3.1. Strains and culture conditions

The strains used in this work are listed in the table 1. The bacterial cells were initially streaked from -80°C glycerol stock and maintained in Luria-Bertani (LB) plates. After growth on LB plates, precultures were initiated from single colonies in 5 mL of LB broth aerobically at 37°C with shaking at 200 rpm for 16 h.

To test if HFDs inhibit the production of virulence factors, overnight cultures were inoculated again in LB broth at initial turbidity of OD_{600} ~1.0. Then HFDs at 10, 50 and 100 µM were added and the cells were cultured for 5 h. DMSO was used as negative control

The bacterial growth was measured using a spectrophotometer (SPECTRONIC GENESYS 5) at 600 nm. After the incubation time, cells were centrifuged and the supernatant was used to determine the expression of QS-controlled virulence factors. For all the assays at least three independent cultures were included.

Table 2. 1. Strains used in this study.

Strain	Relevant genotype/description	Reference
PA14 WT	Laboratory strain originally isolated from a burned patient	Liberati *et al*. 2006
INP-57M	Clinical isolate from cystic fibrosis patients Resistant to synthetic furanone C-30	García-Contreras *et al*. 2015
INP-42	Clinical isolate from cystic fibrosis patients. Multidrug resistant	García-Contreras *et al*. 2015
ΔlasR/rhlR	Mutant with QS systems disrupted	Park *et al*. 2005
Δ*pscC*	Mutant of Type III Secretion System (T3SS)	

2.3.2. Inhibition of quorum sensing controlled virulence factors

P. aeruginosa produces alkaline protease and pyocyanin as virulence factors. They were measured using 2 mL of the supernatants obtained after the 5 hours of incubation treated with the HFDs as previously described. Alkaline protease was detected by the Hide-remazol blue assay and the absorbance was measured at 595 nm (Howe & Iglewski 1984). Pyocyanin was determined spectrophotometrically after its extraction with chloroform and 0.2 N HCl at 520 nm (Essar *et al*. 1990).

The results of specific amounts of alkaline protease and pyocyanin productions were normalized by growth (divided by the O.D. 600 nm of the cultures). In order to calculate the percentage of inhibition, the absorbance of the controls was taken as 100% of alkaline protease or pyocyanin productions (O'Malley *et al*. 2004). DMSO was used as negative control and furanone C-30 as positive control. For all the assays at least three independent cultures were measured.

2.3.3. Inhibition of biofilm formation

The effect of HFDs on biofilm formation was evaluated in polystyrene 96-well plates (Corning®). Overnight cultures of the selected bacteria were diluted (1:100) in LB broth. Wells of the sterile round-bottom 96-well polystyrene plates containing 200 µL of the overnight diluted culture and 10 µL of the treatments at 100, 50 and 10 µM were incubated without shaking during 24 h at 37°C. DMSO was used as negative control and furanone C-30 as positive control. Once the 24 h of incubation, the growth was recorder at 600 nm and the plates were washed three times with distilled water, dried and stained for 20 min with 200 µL of 0.1% crystal violet.

After that the crystal violet was removed and the plates were washed three times with distilled water and dried. For the quantification of attached cells the crystal violet was solubilized with 200 µL of 30% acetic acid in water (O'Toole 2011), and the absorbance was measured at 492

nm (HALO MPR-96) . The formation of biofilms was normalized by growth (divided by the O.D. 600 nm of the cultures). To calculate the percentage of inhibition, the absorbance of the controls was taken as 100% biofilm formation. Data were obtained as an average of three independent experiments with nine replicates.

2.3.4. Swarming motility assay

Swarming motility assay was performed in 6-well plates (Corning®) containing M8 minimal medium supplemented with 1 mM MgSO4, 0.5% glucose, 0.5% casamino acids, and 0.5% agar (Ha *et al*. 2014). HFDs at 50 y 100 µM were added to the motility agar. DMSO was used as negative control and furanone C-30 as positive control. Aliquots (2.5 µL) were taken from overnight cultures and spotted in the center of each well, the migration zones were measured after 24 h of incubation at 37°C. Each assay was performed using three independent cultures per isolate.

2.3.5. Statistical analyses.

The results are presented as the average and standard deviation of at least three independent experiments. The Student's t test for non-paired samples was used for statistical analysis. These analyses were done in IBM-SPSS 22v software. A significance level of $P \leq 0.05$ was used to determine differences between samples.

2.3.6. Mouse abscess model

CD-1 male mice were obtained from General Bioterium of the Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México. The indications of NOM-062-ZOO-1999 for handling and use of laboratory animals, and the Regulation for use and care of animals destined for research at the Colegio de Postgraduados were followed at all times.

The mouse abscess model was performed as previously described (Berube *et al*. 2017; Pletzer *et al*. 2017). Briefly, 6 weeks old CD-1 male mice were shaved and depilating using a

cream hair remover (Nair®). The mice were anesthetized with and intraperitoneal injection of pentobarbital. Prior to the injection the cultures were grown to an OD_{600} ~1.0 in LB broth and the bacterial cells were washed twice with sterile PBS, resuspended and adjusted to $1x10^6$ CFU. Thereafter 60 µL of the bacterial suspension were injected into the subcutaneous space of the right side of the dorsum. The quorum sensing inhibitors (C-30 and GBr) at final concentration of 100 µM (2% final DMSO concentration) were diluted in the bacterial suspension and injected into de subcutaneous space with the bacteria. Gentamicine at 300 µg/mL was used as positive control and PBS as negative control.

The inflammation and dermonecrotic lesion were measured every 24 h during four days. The area was calculated using the following formula:

$$(1/2\ lenght \times 1/2\ width) \times \pi$$

After the four days of postinoculation the livers and the soft tissues containing the inflammation or dermomecrotic area of the mice were excised and homogenized with PBS. Serial dilutions were done in LB plates to count colony forming units (CFU). Experiments were performed at least twice with 5 animals per group.

2.4. Results

2.4.1. Anti-quorum sensing activity in P. aeruginosa strains

Since *P. aeruginosa* produces several virulence factors which are related with the quorum sensing systems, we investigated the alkaline protease and pyocyanin production in presence of different HFDs. Notably GBr reduces the pyocyanin production up to 70% in all strains in a dose-dependent manner (Figure 2.2E) and more than the positive control (C-30), but at 100 µM the bacterial growth was affected in the strain PA14 by these compounds (Figure 2.2A).

Low concentrations of C-30 and GBr (50 and 10 µM) didn't affected the cell growth of the strains (Figure 2. 2 A-C) and still inhibited the production of virulence factors. Also in the isolates INP-57M and INP-42 the Bro. A compound strongly reduced this virulence factor (Figure 2. 2E).

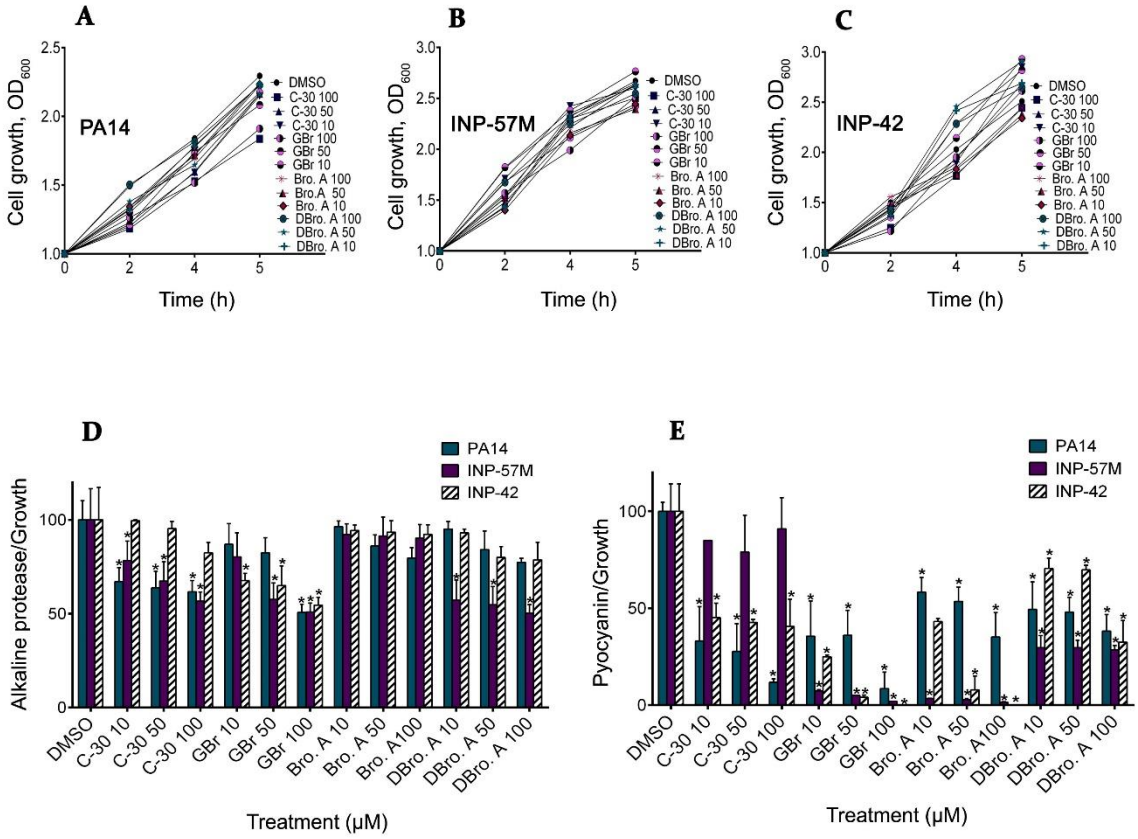

Figure 2. 2. Antivirulence activities of HFDs in *P. aeruginosa* strains. (A), (B) and (C) Cell growth. (B)Alkaline protease production. (C)Pyocyanin production. At least three independent experiments were conducted. (*P<0.05)

The different DEXT compounds didn´t show significantly inhibition of pyocyanin production (Figure 2.3E), but DEXT 1, 2 and 4 reduced the alkaline protease production up to 30% in the three isolates (Figure 2. 3D) as well as GBr in INP-57M and INP-42(Figure 2. 3D), without affecting the bacterial growth (Figure 2. 3A-C).

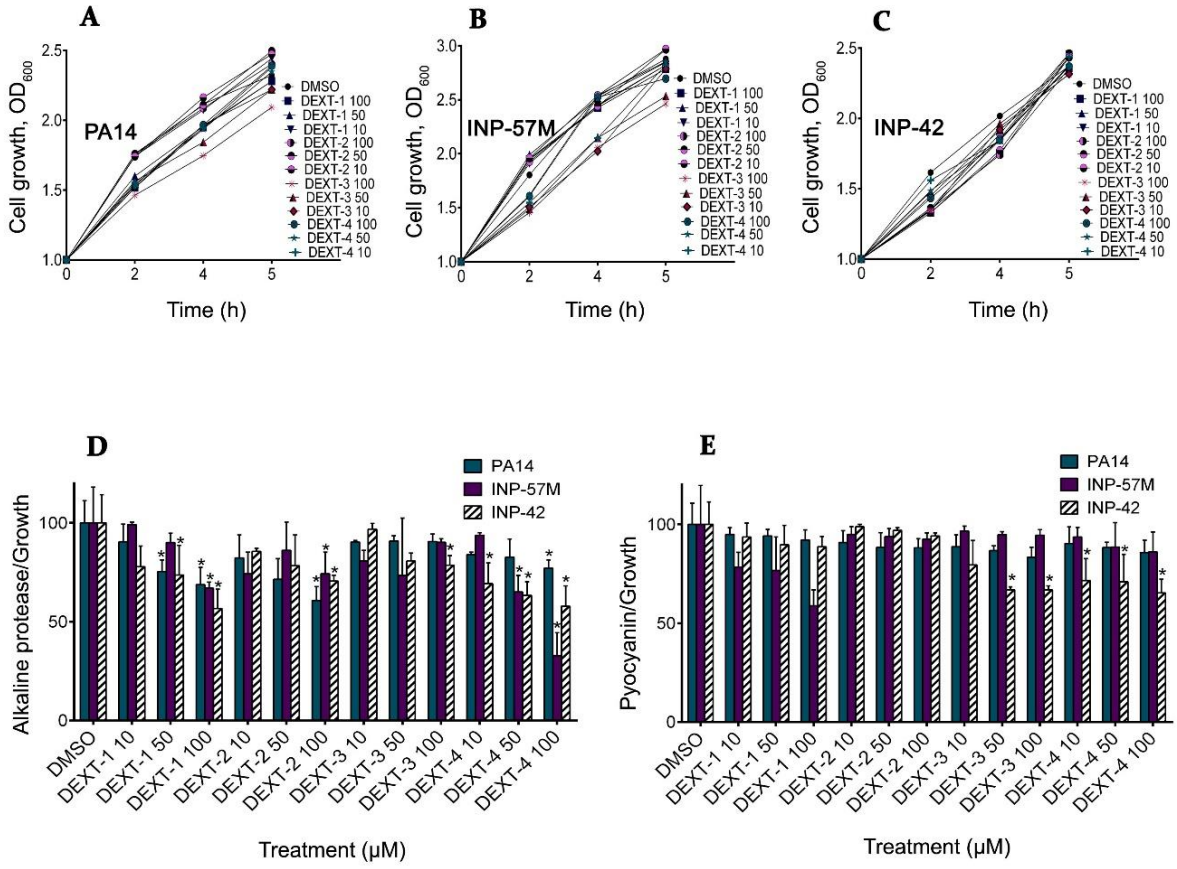

Figure 2. 3. Antivirulence activities of DEXT halogenated compounds in *P. aeruginosa* strains. (A), (B) and (C) Cell growth. (D) Alkaline protease production. (E) Pyocyanin production. At least three independent experiments were conducted. (*P<0.05).

2.4.2. Effects of HFDs on biofilm formation

All HFDs tested showed significant and dose-dependet inhibition of biofilm formation in the strains (Figure 2.4). The molecules C-30, GBr, DEXT-1 and DEXT-2 decreased biofilm formation by more than 40-50% in the strains PA14 and INP-42. The different compounds showed less inhibition effects against the isolate INP-57M (Figure 2.4A and B). Interestingly only the DEXT compounds at the highest doses (50 and 100µM) have noticeably inhibition effects in the resistant strain INP-42 (Figure 2.4B).

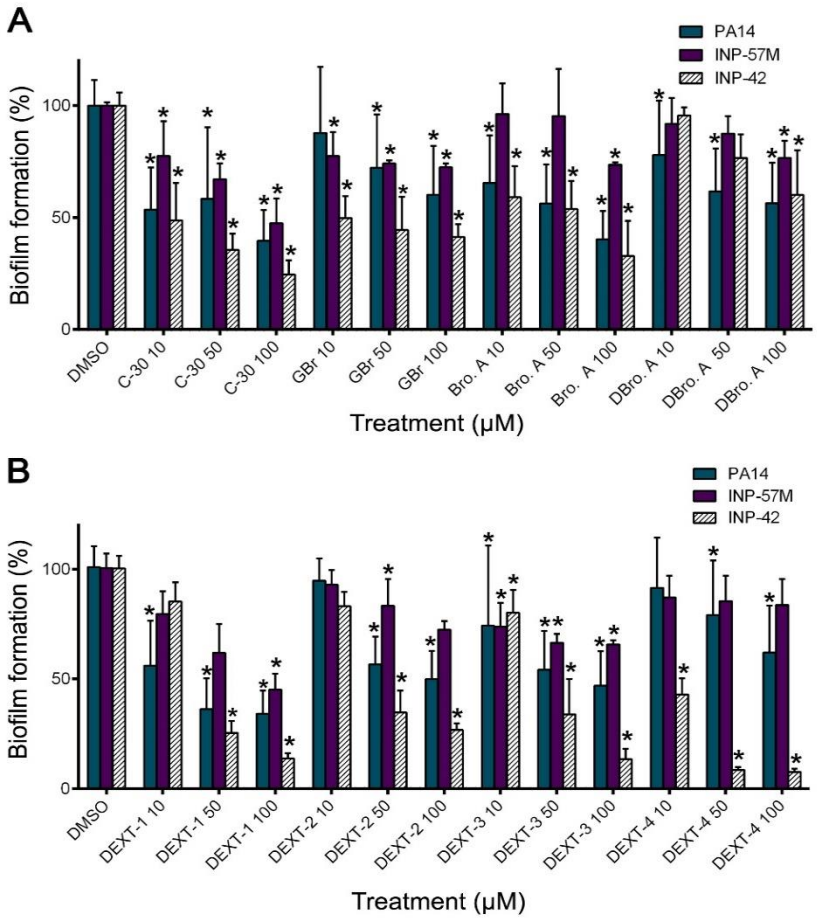

Figure 2. 4. Quantification of biofilm formation by *P. aeruginosa* strains in presence of HFDs (A) and (B) on biofilm formation against different *P. aeruginosa* strains. (*P<0.05)

2.4.3. Effects of HFDs on swarming motility

The majority of the molecules almost abolished the swarming motility at 100 and 50µM in the three isolates (Figure 2. 5A and B). In the case of strain PA14, the compounds C-30, GBr and DEXT-3 at 100 µM affected the bacterial growth but at 50µM the motility still was inhibited without growth effects (Figure 2. 5A and B).

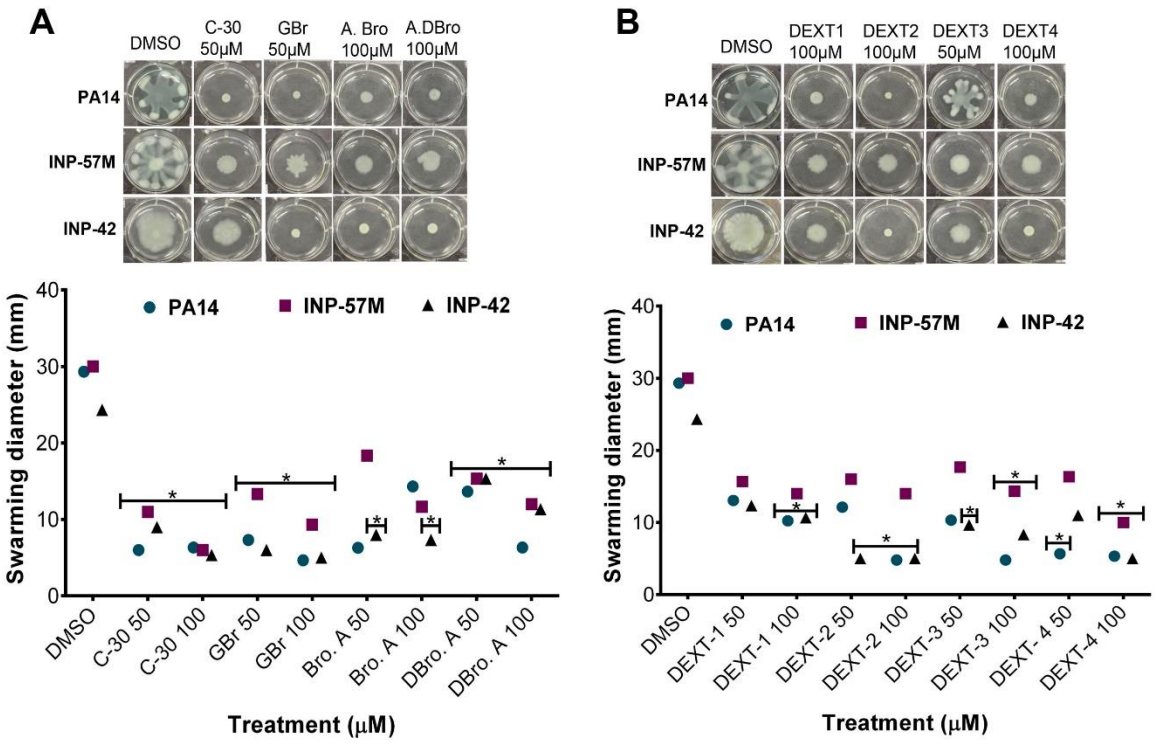

Figure 2. 5. Swarming motility of the *P. aeruginosa* strains in presence of HFDs (A) and (B). The values (means) that are significantly different from the control group are shown by a bar and asterisk ($P<0.05$). The pictures above the graph show representative images of the inhibition at 50 or 100µM by the compounds.

2.3.4. Effect of C-30 and GBr inhibitors on the PA14 mouse subcutaneous abscess model

Previous reports showed that *P. aeruginosa* is capable of forming abscesses after subcutaneous injection of PA14 at $1x10^6$ CFU (Berube *et al*. 2017; Pletzer *et alk* 2017). Using this mouse model we evaluated the protective effect of the C-30 and GBr compounds at 100µM against abscess formation, since they were the most effective inhibitors of the virulence factors of the *P. aeruginosa* strains in the previous experiments.

Two additional strains of *P. aeruginosa* were used in this study: Δ*lasR/rhlR* a mutant in which the quorum-sensing regulators LasR and RhlR are deleted, and the Δ*pscC* a mutant in which

the T3SS apparatus is not well assembled. These two strains were used as negative controls since are incapable to express virulence factors necessary to form abscess.

Subcutaneous injection of the PA14 wild type strain lead to the formation of an abscess and reaches its peak size of inflammation within the first 24 h (Figure 2. 6A). After this time the abscess caused dermonecrotic lesions at 2 days of postinoculation (Figure 2. 6B). As expected, the strains Δ*lasR/rhlR* and Δ*pscC* did not cause abscess or dermonecrotic lesions (Figure 2. 6A and B)

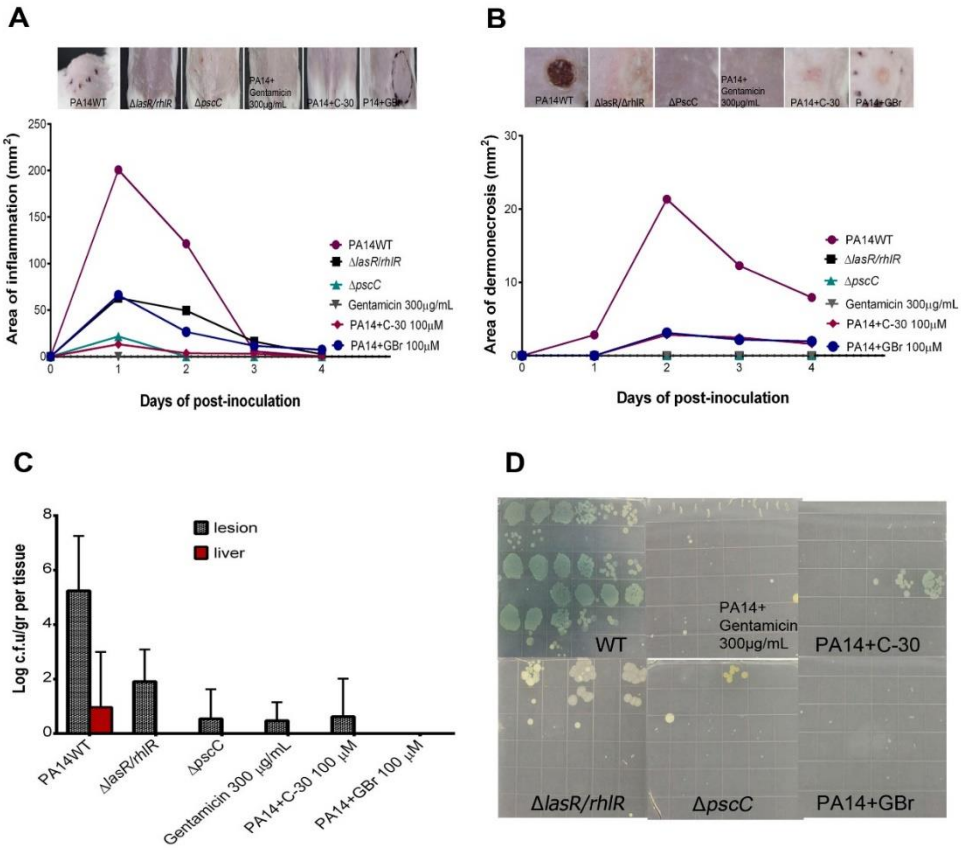

Figure 2. 6. Infection of *P. aeruginosa* and therapeutic treatment in the subcutaneous model. (A) Area of inflammation. (B) Area of dermonecrosis. The figures above are the graphs are representative photographs of inflammation and dermonecrosis in the abscess. (C) c.f.u in lesion and livers. (D) Culture plates of the tissues. Five mice were used per group. c.f.u., colony forming units.

The administration of C-30 and GBr led to the formation of a significantly smaller abscess and dermonecrotic lesions than PA14 and were similar to the effect showed by the antibiotic (Figure 2.6A and B). Furthermore, the mice with PA14 exhibited a high bacterial burden in the lesion site and liver (Figure 2.6C y D) and those treated with gentamicin, C-30 and GBr not exhibited bacterial dissemination and lower bacterial burden in the abscess (Figure 2.6C y D).

To corroborate that the protective effect shown by C-30 and GBr was not due to a bactericidal effect, after 15 minutes of addition of the compounds to the bacterial solution, the bacteria were counted by serial dilution; the results demonstrated bactericidal effect of C-30 and GBr at 100µM.

2.4. Discussion

The identification and synthesis of new compounds with the capability to inhibit bacterial QSS are a promising strategy known as quorum quenching (QQ) to combat multidrug-resistant bacteria. In contrast with the mode of action of antibiotics, QQ molecules are compounds directed against unconventional targets like the inhibition of virulence factors without compromising the bacterial survival.

The QQ activity of the synthetic brominated furanone (SBF) C-30 against virulence factors in *P. aeruginosa* has been widely reported (Hentzer *et al.* 2003; Wu *et al*. 2004; Maeda *et al*. 2012). This inhibitor is a derivate of the halogenated furanones (HFs) produced by the marine macro-alga *Delisea pulchra*. Here, we report the QQ activity of new derivatives of HFs with structures related to these inhibitors (Figure 1) against three *P. aeruginosa* strains: reference strain PA14 and two resistant clinical isolates(Table1)

The results showed that the QQ activity of the HFDs against pyocyanin and alkaline protease is variable, pyocyanin being the substance most easily inhibited by the compounds. The furanone C-30 repressed alkaline protease and pyocyanin production in the reference strain PA14 but was less active in the clinical strains (INP-57 and INP-42). This may be related to previous results that showed resistance to C-30 of these strains (García-Contreras *et al*. 2015).

The GBr was the most effective compound inhibiting pyocyanin and alkaline protease in all strains, but similarly to C-30, at 100µM it inhibited cell growth in strain PA14. Although the structure of GBr is closely related to C-30 (Figure 1), in the strains INP-57M and INP-42 it also showed important inhibitory activity.

P. aeruginosa readily causes acute and chronic infections in immunocompromised patients and their capacity to form biofilms renders their eradication by the immune systems and antibiotics ineffective (Rasamiravaka *et al*. 2015). Importantly, a direct link between biofilm formation and bacterial motility has been reported, since swarming motility is implicated in the early stages in biofilm establishment (Daniels *et al.* 2004).

All the HFDs tested notably decreased the biofilm formation and swarming motility specially in the clinical strains INP-57M and INP-42. This demonstrates its potential use in biomedical and food applications because another important target related with QSS is controlling the early steps of bacterial adhesion, essential for the establishment of infection and colonization in general (Musk & Hergenrother 2006; de Lima Pimenta *et al*. 2013). In literature, the inhibition of biofilm formation and swarming motility by other halogenated furanones has also been reported (Maeda *et al*. 2012; de Lima Pimenta *et al*. 2013; Shetye *et al*. 2013).

To date three interrelated QS systems in *P. aeruginosa* are known, the LasI-LasR and RhlI-RhlR systems with acil-homoserin lactones (AHLs) as autoinducers; and the PQS system in which

a quinolone is the signal molecule (Wu *et al*. 2004; Jimenez *et al*. 2012; Lee & Zhang 2015). Particularly, synthetic BFs act as antagonist molecules, interfering with the recognition of autoinducer by the transcriptional regulator (Rasmussen & Givskov 2006; Defoirdt *et al*. 2010).

The furanone C-30 interferes with recognition in the *lasR* system implicated on biofilm formation and swarming motility (Hentzer *et al*. 2003; Janssens *et al*. 2008). The HFDs with closely related structures to C-30 probably act as antagonist. Further studies should explore this hypothesis.

In the clinical strains INP-57 and INP-42 the HFDs notably decrease pyocyanin production, biofilm formation and swarming motility. The use of clinical strains for the evaluation of potential QQ is crucial, as their resistance to compounds like C-30 and 5-fluorouracil by efflux pumps or by the decrease in the compound uptake has been demonstrated (Maeda *et al*. 2012; García-Contreras *et al*. 2013). This limits their effectiveness against all the strains present in infections (Kalia *et al*. 2014; García-Contreras 2016).

Another important aspect to consider in the evaluation of the efficacy of QQ is the use of adequate animal infection models that determine the potential of compounds for therapeutic use against bacterial infections more rapidly (Berube *et al*. 2017). Some mouse models have been proposed simulating lung and cystic fibrosis infections, as well as the thermally-induced injury model in which diverse QQ like azithromycin, halogenated anthranilic acids analogs and synthetic halogenated furanones decrease mouse mortality but the infection was not eradicated (Wu *et al*. 2004; Hoffmann *et al*. 2007; Lesic *et al*. 2007).

However, these models present various problems like inconsistency, lethality or rapid clearance depending on the infecting doses and in some cases are highly invasive. The mouse abscess model has been proposed to test the antivirulence compounds in *P. aeruginosa* infection,

and has the advantage of being reliable and reproducible (Berube *et al*. 2017; Pletzer *et al*. 2017). This was confirmed in or experiments.

In this model, our results showed that C-30 and GBr decrease the formation of abscess and dermonecrotic lesions, but it seems due to a bactericidal effect in the dose used. The use of different doses and assessment of the toxicity of the compounds is very important, to confirm that the positive effects observed in the *in vivo* models are a result of quorum sensing inhibition or bactericidal action (Defoirdt *et al*. 2013; García-Contreras 2016). Future assays with other concentrations of HFDs should be used to corroborate their QQ in the mouse model.

Although some HDFs assayed demonstrated growth inhibition, synthetic compounds have the advantage that they can be further modified to obtain non-bactericidal agents (Shetye *et al*. 2013). Also these QQ can be tested in conjunction with antibiotics and other inhibitors with targets implicated in bacterial pathogenesis and not regulated by QS like type III secretion system (T3SS) to broaden their usefulness in combating bacterial infections (Berube *et al*. 2017; Muñoz-Cazares *et al.* 2018).

Future work will focus on the effect of the HDFs in non-toxic concentrations and in combination with other inhibitors in the mouse abscess model to corroborate their efficacy as antivirulence therapy to combat multidrug-resistant bacteria.

2.5. Conclusions

This study demonstrated the quorum quenching activity of newly synthetized HDFs. Further work needs to assess the toxicity at different doses in the mouse model, to confirm that the results obtained are related to the inhibition of QS. Also the compounds could potentiate other virulence inhibitors to render *P. aeruginosa* less virulent.

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CHAPTER III. PHENOLIC COMPOUNDS WITH ANTI-VIRULENCE PROPERTIES

Muñoz-Cazares N, García-Contreras R, Pérez-López, Castillo-Juárez I. 2017. Phenolic compounds with anti-virulence properties. In: Soto-Hernández M, Palma-Tenango M, García-Mateos MR, eds. *Phenolic Compounds - Biological Activity*. INTECH, 139-167.

Phenolic compounds with anti-virulence properties

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3.1. Abstract

Natural products represent the major source of approved drugs and still play an important role in supplying chemical diversity as well as new structures for designing more efficient antimicrobials. They are also the basis for the discovery of new mechanisms of antibacterial action. In this regard, a large number of substances, mainly extracts from natural sources, have been obtained in order to identify their anti-virulence activity. In recent years, there is an increase in the study of anti-virulence natural product derivatives. Different targets have been proposed as a solution to the serious problem of bacterial antibiotic resistance. Inhibition of bacterial quorum-sensing systems has been one of the most studied; however, there are other mechanisms involved in virulence regulation, damage to the host and bacterial survival, which suggests that there are another good targets such as bacterial secretion systems, biofilm formation, two-component systems, flagellum, fimbriae, toxins and key enzymes. Within the natural products, the main anti-virulence compounds

are phenolic in nature, so that the next chapter describes and analyzes the relationship between chemical structure and activity of the main phenolic compounds reported.

Keywords: anti-virulence, antibiofilms, antibiotic resistance, phytochemicals, quorum sensing,

3.2. Introduction

Since their introduction in the middle forties, antibiotics had been extensively used for the treatment of infectious diseases, producing remarkable results and saving millions of lives worldwide (Aminov 2010); nevertheless, bacteria are very dynamic organisms able to interchange genes by several mechanisms including conjugation, transformation and transfection via bacteriophages (Aminov 2010).

In addition, they usually replicate at high rates and hence have the ability to evolve quickly and adapt to strong selective pressures; this combined with the selfprescription, inadequate prescription by some physicians (e.g., to treat viral diseases) and their improper use by patients who do not complete the recommended treatment scheme has derived in an alarming situation since to date antibiotic resistance (including multiresistance and panresistance) is a common trend in most of hospital-acquired infections and is becoming more common in community-acquired ones (Spellberg *et al*, 2008; López-Pueyo *et al*. 2011).

In fact, the situation is so delicate that recently, the OMS warned that if the current trends are still observed, then by the year 2050 we will enter the post-antibiotic era and previously treatable infectious diseases will cause more deaths than other important diseases such as cancer (Aryee & Price 2015).

Hence, the discovery of new antibiotics as well as the development of alternative approaches to combat bacterial infections is urgently needed (Rangel-Vega *et al*. 2015); among such new approaches are the inhibition of bacterial antibiotic resistance mechanisms, the

utilization of non-antibiotic bactericide agents such as bacteriophages, the repurposing of clinically approved drugs, and the inhibition of bacterial virulence (Rangel-Vega *et al*. 2015). For the first approach, already successful examples can be found in the clinic; by instance, the co-utilization of clavulanic acid (an inhibitor of β-lactamases) and amoxicillin is commonly administrated (Finlay *et al.* 2003); and current research is focused on the utilization of broad spectrum anti-resistance compounds such as those inhibiting multidrug efflux pumps (Tegos *et al.* 2011).

Regarding the second approach, it was recently demonstrated that some anticancer drugs such as 5-fluorouracil (Ueda *et al*. 2009), mitomycin C (Kwan *et al*. 2015) and cisplatin (Chowdhury *et al*. 2016) have remarkable antibacterial properties, while bacteriophages had been used in east European countries for the treatment of diverse bacterial infections, and currently, its utilization in the occidental medicine is being proposed (Abedon *et al*. 2011; Young & Gill 2015).

Finally, targeting bacterial virulence instead of their viability is a concept that had derived in several publications, mostly centered in the inhibition of master virulence regulators such as quorum-sensing (QS) systems, which allow several Gram-negative and Gram-positive bacteria to coordinate the production of several virulence factors, once a high population density is reached (Figure 3.1A).

Indeed, initially, it was claimed that this approach will be impervious to the generation of resistance since *in vitro* in rich media QS does not control metabolic processes linked to growth; nevertheless, in some conditions, QS inhibition can promote resistance (Maeda *et al*. 2012; García-Contreras *et al.* 2013a; García-Contreras 2016) and not all clinical strains are sensitive toward current QS inhibitors (García-Contreras *et al*. 2013b).

However, since QS also regulates the stress response, it has been shown that QS-inhibited bacteria are more susceptible to the action of disinfectants, antibiotics and the immune system (Bjarnsholt *et al*. 2005; García-Contreras *et al*. 2014), and hence, QS inhibition may be a valuable adjuvant therapy for recalcitrant bacterial infections (García-Contreras *et al*. 2016).

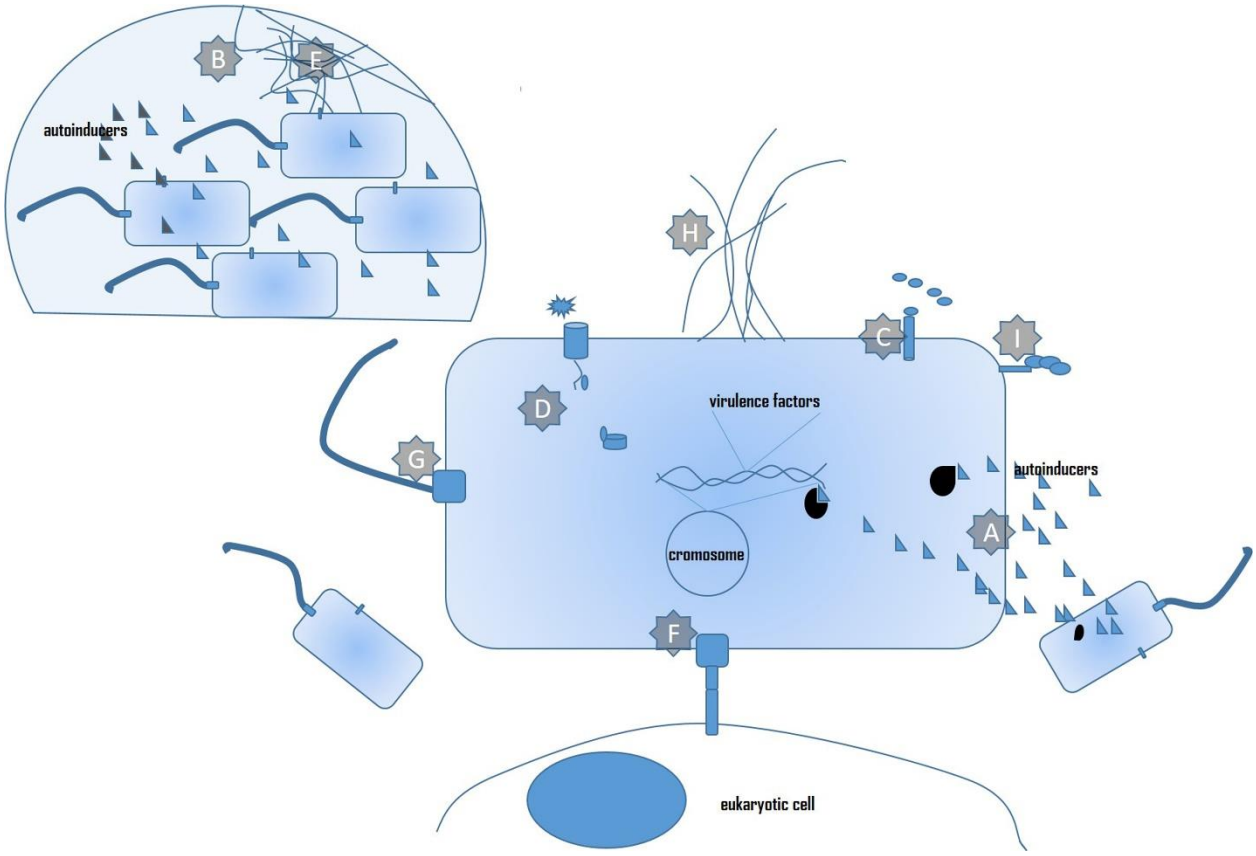

Figure 3. 1. Main targets of anti-virulence of phenolic compounds. A: Quorum-sensing system, B: biofilm formation, C: toxins, D: two-component systems, E: curli fibers, F: bacterial type III secretion systems, G: flagellum, H: fimbriae, I: sortase enzymes.

Another key factor for the development of chronic infections and colonization of surfaces is the formation of biofilms, which is the main way the bacteria are found in nature (Padilla-Chacón *et al*. 2017).

As mentioned previously, QS is a master regulator of the production of several bacterial virulence factors, such as: exoproteases that degrade connective tissue such as elastase and alkaline protease (collagenase), phenazines that promote the generation of reactive oxygen species, siderophores that facilitate iron uptake, toxins that disrupt cellular processes and exopolysaccharides that form phagocytosis-resistant capsules and participate in the generation of the biofilm matrix (Castillo-Juarez *et al*. 2015) (Figure 3.1C).

These structures consist of multicellular communities enclosed in a matrix which makes them extremely resistant to antibacterial agents (Figure 3.1B) (Castillo-Juarez *et al*. 2017). They also provide robust niches that allow the bacteria to protect themselves from environmental fluctuations and against the immune system, which drastically reduces the effectiveness of antimicrobial therapy (Padilla-Chacón *et al*. 2017).

Since for many pathogenic bacteria QS is the main regulator of expression of bacterial virulence factors, its disruption has been the main anti-virulence strategy investigated to date (Castillo-Juarez *et al*. 2015). However, another alternative that has also been reported is the direct inhibition of individual virulence factors, such as toxins, response regulators (two-component regulatory systems (TCS) and processes involved in the formation and maturation of structures such as the curli, the bacterial type III secretion system (T3SS), fimbriae and flagellum.

TCS are response regulators which are formed by a protein localized in the cytoplasmic membrane called histidine kinase sensory protein (HKSP), which acts as an environmental sensor that is activated in ATP-dependent way (Figure 3. 1D) (Mitrophanov & Groisman 2008). HKSP then activates a response regulator protein (RRP) found in the cytoplasm which is responsible for recognizing DNA sequences that modulate the expression of genes involved in various functions such as chemotaxis, porin expression and expression of virulence factors among others (Figure 3.

1D) (Mitrophanov & Groisman 2008). An important feature is that TCRs have not detected in mammalian cells, so there are a suitable specific target to treat bacterial infections (Gotoh *et al*. 2010).

The curli (Figure 3.1E) is the major protein component of the extracellular matrix and is mainly produced by enterobacteria to aid in the formation of three-dimensional structures such as biofilms (Costerton *et al*. 1995). Curli fibers belong to a growing class of fibers known as amyloid fibers, which are also involved in host cell adhesion and invasion, and are also strong inducers of host inflammatory response (Costerton *et al*. 1995). The structure and biogenesis of curli are unique among bacterial fibers and represent an excellent anti-virulence target (Barnhart & Chapman 2006).

The type III secretion system (T3SS) also known as the injectisome is a multiprotein apparatus that facilitates the secretion and translocation of toxins or effector proteins from the bacterial cytoplasm directly to eukaryotic cells (Figure 1F) (Aiello *et al*. 2010; Gu *et al*. 2015). It is highly conserved in most Gram-negative pathogens, but its presence is not a necessary condition for bacterial survival *in vitro* (Gu *et al*. 2015).

Motility and recognition surfaces are key factors for the dispersal and colonization of new niches by bacteria (Erhardt 2016). For that, the flagellum and the fimbriae are target structures suitable for anti-virulence molecules (Knight & Bouckaert 2009; Erhardt 2016). The flagella (Figure 3.1G) are multiprotein complexes based on flagellin, which rotate allowing bacterial displacement in aqueous media (Knight & Bouckaert 2009), while fimbriae (Figure 3.1H) are extracellular protein structures mainly constituted by pilin, which start in the plasma membrane, cross the cell wall and extend around the cell. These structures allow the adhesion of bacteria mainly to epithelial cells (Hendrickx *et al*. 2011).

Another important virulence factors are the sortase enzymes (cysteine transpeptidases) (Figure 3.1I), which are used by Gram-positive bacteria to display proteins in cell surface, such as glycoproteins (Hendrickx *et al*. 2011) , and they can also attach to proteins in the cross-bridge peptide of the cell wall or link other proteins together to form pilin (Spirig *et al*. 2011). The phenomenon of protein deployment is essential for the development of virulence factors and promotes nutrient acquisition, adhesion and immune system evasion (Hendrickx *et al*. 2011). Because surface proteins play a fundamental role in microbial physiology and are frequently virulence factors, sortase enzymes are a very important target (Spirig *et al*. 2011).

Reports related to the study of natural products as anti-virulence molecules had increased in the last decade. Their powerful attack against bacterial infections without promoting resistance and the elimination of antibiotic-resistant strains are the most attractive features of this kind of compounds. Among natural products with anti-virulence activity, those derived from plants with anti-QS and antibiofilm activity are the most common (Martín-Rodríguez *et al*. 2016).

Phenolic compounds are secondary metabolites present in plants, which are crucial in many aspects of their lives, especially during the interactions with the environment, since they are used in the defense of plants against bacterial pathogens. Similarly, compounds of phenolic type are the major metabolites with anti-virulence properties described so far, and specifically, the flavonoids are the main representatives (Silva *et al*. 2016).

Most of the biologically active reported phenolic compounds have chemical structures with previously identified antimicrobial, antioxidant and anticancer activity. Similarly, for some of them their participation in the regulation of various physiological functions in plants and animals is well known. In recent years, the anti-virulence properties of phenolic compounds are being

unravel, and most of the cases depend on the compound concentration and the bacterial system in which the phenolic compounds can exhibit bactericidal or anti-virulence effects.

In the next chapter, we discuss studies of phenolic compounds derived mainly from plant species, starting with those that are better characterized and that have more anti-virulence reported properties. We focus on the relationship between their structures and their activity.

3.3. Phenolic compounds anti-virulence

3.3.1. Epigallocatechin gallate and related compounds

It is well documented that this kind of compounds has antimicrobial, antioxidant, anti-inflammatory, hypocholesterolemic and cancer-preventive properties (Mitscher *et al*. 1997; Niedzwiecki *et al*. 2016). The *epigallocatechin gallate* (EGCG) (Figure 3.2A) is one of the flavonoids with the largest number of reports related to its antibiofilm activity; remarkably high compound doses can inhibit bacterial growth, but sublethal concentrations exhibit anti-virulence properties.

At the same concentration, catechin (Figure 3.2B) and EGCG inhibit the formation of biofilms of *P. aeruginosa*; however, only *catechin* do not affect the growth (Jagani *et al*. 2009), so the presence of galloyl group in EGCG seems to favor the bactericidal effect. In this regard, it is suggested that EGCG affect the viability because it binds to peptidoglycan, hence directly disrupting the integrity of the bacterial cell wall. Similarly, EGCG at concentrations that affect bacterial viability inhibit the biofilm of *Enterococcus faecalis*, an opportunistic pathogen implicated in urinary tract infections, endocarditis and root canal infections (Lee & Tan 2015). In this case, biofilm inhibition is attributed to a bactericidal effect, where the EGCG induces hydroxyl radicals that can damage DNA, proteins and lipids (Lee & Tan 2015).

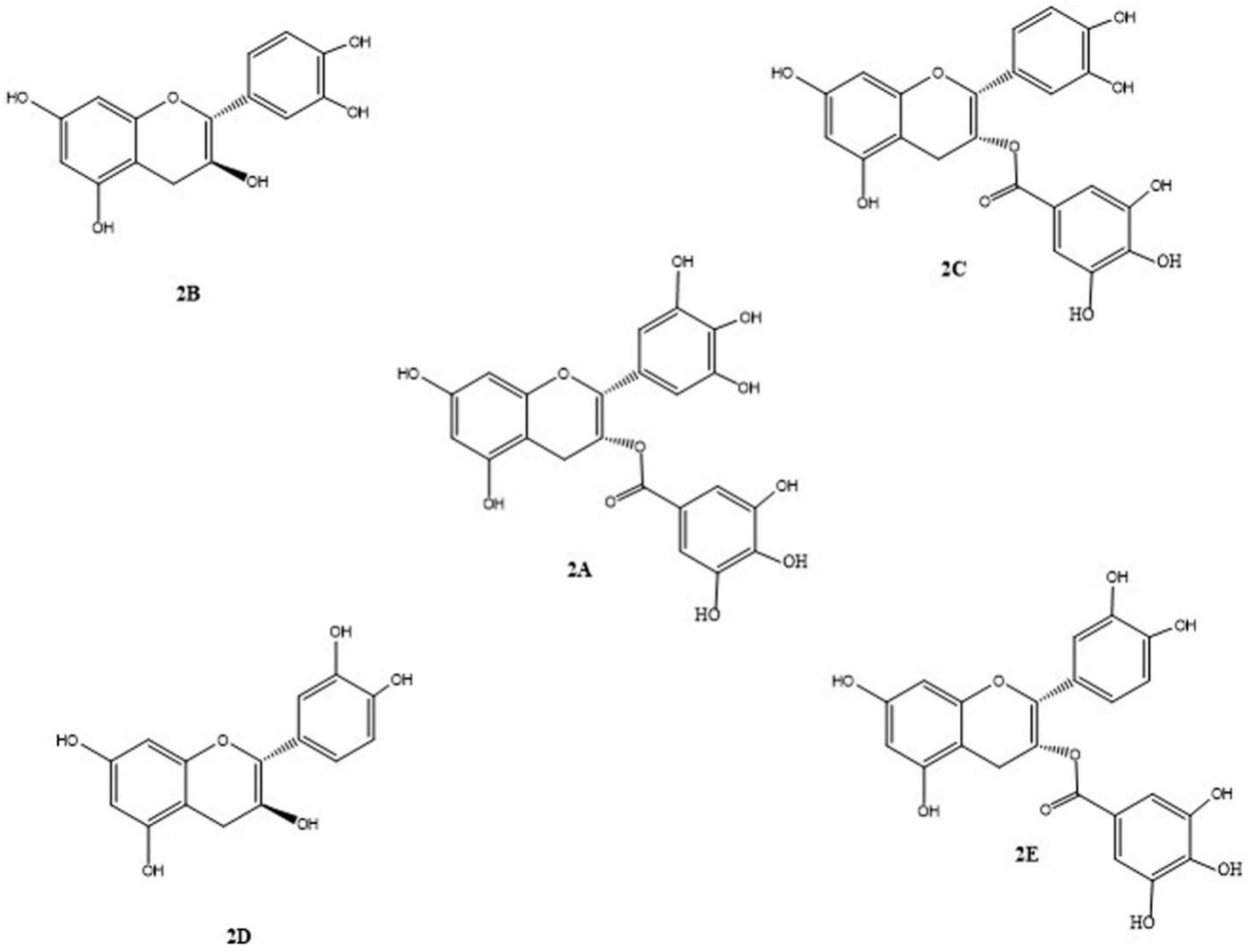

Figure 3.2. Epigallocatechin gallate and related compounds with anti-virulence properties. A: Epigallocatechin gallate, B: catechin, C: catechin-gallate, D: catechin-gallate, E: (−) epicatechin gallate.

However, using sublethal concentrations, it has been found that EGCG significantly decreased the expression of virulence genes that regulate the expression of cytolysins, gelatinase and serine protease in *E. faecalis* (Lee & Tan 2015). It also inhibits biofilm formation of Staphylococcal isolates by interfering directly with polysaccharides of the glycocalyx (Blanco *et al*. 2005). Similarly, it inhibits swarming and biofilm formation of *Burkholderia cepacia* without affecting the growth, likely through QS inhibition (Huber *et al.* 2003).

EGCG and *catechin gallate* (Figure 3.2C) directly inhibit the anthrax lethal factor (LF) produced by *Bacillus anthracis*, which has a key role in the development of anthrax (Dell'Aica *et al*. 2004). LF is a zinc metalloprotease that directly affects MAPK-signaling kinases, which are essential for transmitting signals in eukaryotes. EGCG and *catechin gallate* block the activity of LF, preventing MAPK-kinases cleavage and macrophages death (Dell'Aica *et al*. 2004). In the case of EGCG, it also delays the death of mice exposed to the anthrax toxin (Dell'Aica *et al*. 2004). It is noteworthy that although other catechins were evaluated, the presence of a galloyl group in the structure seems to be essential for this anti-virulence activity.

For the case of *catechin* (Figure 3.2B), it has also been reported that it inhibits the production of virulence factors regulated by QS in *P. aeruginosa*, such as pyocyanin and elastase (Vandeputte *et al*. 2010). Also, it was found to have a negative impact on the transcription of several genes involved in QS, such as those codifying proteins involved in the synthesis of autoinducer molecules (Vandeputte *et al*. 2010).

Dental plaque is a complex biofilm that allows the survival and development of *Streptococcus mutans*. It has been reported that EGCG shows bactericidal activity against *S. mutans*; in addition, its antibiofilm activity is due to reducing the adherence of bacteria to surfaces by direct inhibition of glucosyltransferases (Xu *et al*. 2011), which are enzymes that synthesize polysaccharides (Hattori *et al*. 1990; Nakahara *et al*. 1993).

However, at sublethal concentrations, EGCG reduces biofilm by interfering with gene regulation, specifically by inhibiting the expression of the *gtf* genes (encoding glucosyltransferases), which are associated with adhesion and formation of biofilms (Xu *et al*. 2012). Moreover, it represses genes encoding virulence factors associated with acidogenicity and

acidurity, such as *ldh*, *eno*, dATP, Agud and the activity of the F_1 F_0 -ATPase and lactate dehydrogenase (Xu *et al*. 2011).

EGCG at sublethal concentrations also inhibits motility and biofilm formation of *Campylobacter jejuni*, a foodborne pathogen which is one of the main causes of gastrointestinal infections worldwide (Castillo *et al*., 2015). In this case, the mechanism involved in biofilm inhibition is related to QS inhibition (Castillo *et al*. 2015).

It is worth noting that to date there are no studies to investigate its structure-activity relationship, so it is not yet known which parts of the structure are critical to their anti-virulence effects. However, for the *(−) epicatechin* (Figure 3.2D) which also possesses anti-QS activity against *Chromobacterium violaceum*, a Gram-negative bacteria with AHLs mediated QS (Borges *et al.* 2013).

The *(−) epicatechin gallate* (Figure 3.2E) at sublethal concentrations inhibits two of the major determinants of virulence in *S. aureus*, the α-toxin and the coagulase (Shah *et al*. 2008). Furthermore, it has been shown that in combination with β-lactams, it is efficient to eliminate multiresistant strains of *S. aureus*. Although it has been observed that some synthetic analogs have better pharmacokinetic properties than the native *(−) epicatechin gallate* (Anderson *et al*. 2005a, 2005b).

3.3.2. Cinnamaldehyde and related compounds

Cinnamaldehyde(CN) (Figure 3.3A) is a major constituent of cinnamon essential oils and occurs naturally in the bark and leaves of cinnamon trees of the genus *Cinnamomum* (Jia *et al*. 2011). The antimicrobial activity of this compound has been proven (Bowles *et al*. 1995; Gupta *et al*. 2008), but new studies have explored their anti-virulence properties, and in contrast to another

compounds, it is considered a nontoxic substance widely used in food and in the cosmetic industry and their use is generally recognized as safe (Amalaradjou *et al*. 2010).

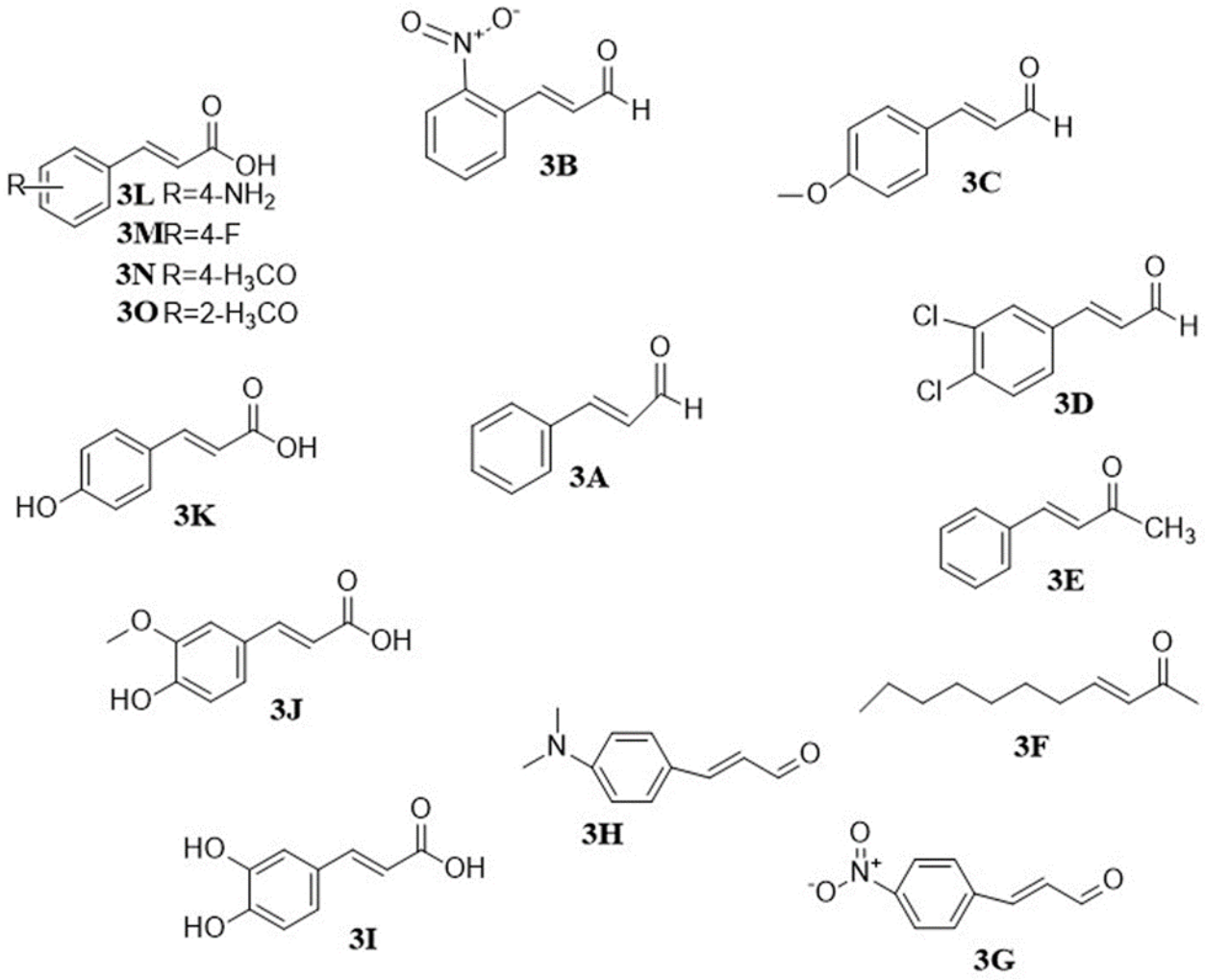

Figure 3.3. Cinnamaldehyde and related compounds with anti-virulence properties. A: Cinnamaldehyde, B: 2-nitrocinnamaldehyde, C: 4-methoxy-cinnamaldehyde, D: 3,4-dichloro-cinnamaldehyde, E: (E)-4-phenyl-3-buten-2-one, F: (E)-3-decen-2-one, G: 4N-4-nitrocinnamaldehyde, H: 4D-4-dimethylaminocinnamaldehyde, I: caffeic acid, J: ferulic acid, K: p-coumaric acid, L: TS027, M: TS110, N: 4-methoxy-cinnamic acid, O: trans-2-methoxy-cinnamic acid.

In *P. aeruginosa*, the acylated homoserine lactones (AHLs) are their main autoinducer molecules (Figure 3.1A) and the CN can inhibit their synthesis as well as the production of the phenazine, pyocyanin and swarming motility (Chang *et al*. 2014). Remarkably, CN also has antitoxin production and anti-hemolytic activities (Kim *et al*. 2015). Similarly, in *C. violaceum*, *Yersinia entrerolitica* and *Erwinia carotovora*, the concentration of AHLs was also reduced by CN and the mechanism proposed was the inhibition of synthesis or degradation transformation of the autoinducer (Truchado *et al*. 2012).

The antibiofilm properties of CN have been widely documented; for example, in *P. aeruginosa* and in enterohemorrhagic *Escherichia coli*, this compound markedly abolished the biofilm formation in a dose-dependent manner by reducing the swarming motility and fimbriae production, respectively. In a previous report, it was shown that for the uropathogenic *E. coli*, CN prevented biofilm formation on plates and catheters, furthermore effectively inactivated preformed biofilms (Amalaradjou *et al*. 2010).

The mechanism proposed for the biofilm inhibition was related to the hydrophobicity of this compound, which helps to target lipids located in the bacterial cell membrane and mitochondria, increasing the membrane permeability, leading to the leakage of ions and other cell contents (Sikkema *et al.* 1994; Amalaradjou *et al*. 2010). The foodborne pathogen *Listeria monocytogenes* forms biofilm for persistence and survives in which CN has inhibitory effect on formation and inactivating mature biofilm by means of the down-regulated critical genes for biofilm formation in this bacteria (Upadhyay *et al*. 2013).

In *Vibrio harveyi*, the autoinducer-2 (A2) is also blocked by CN in a concentration-dependent way by decreasing the binding ability of the autoinducer to its response regulator

protein. Between cinnamaldehyde derivatives, the *2-nitro-cinnamaldehyde* (Figure 3.3B) was he most active compound yielding an inhibition of A2 similar to CN (Brackman *et al*. 2008).

Similarly, the *2-nitro-cinnamaldehyde* and *4-methoxy-cinnamaldehyde* (Figure 3.3C) inhibit pigment production and protease activity in *Vibrio anguillarum* (Brackman *et al*. 2008). The CN is an aromatic carboxylic acid, and its inhibitory was highly dependent on the substitution pattern of the aromatic ring. Replacement of the dimethylamine (Me_2N) substituent with a methoxy (MeO) or a nitro (NO_2) group enhanced the activity (Brackman *et al*. 2008).

Various cinnamaldehyde analogs were also evaluated against *Vibrio* spp. The most active compounds were *2-nitro-cinnamaldehyde*, *3,4-dichloro-cinnamaldehyde* (Figure 3D), *(E)-4-phenyl-3-buten-2-one* (Figure 3.3E) and (*E)-3-decen-2-one* (Figure 3.3F), which show inhibitory activity in A2, bioluminescence, pigment and protease production (Brackman *et al*. 2011). In this case, also the inhibitory effect of cinnamaldehyde analogs was dependent on the structure, and analogs in which the aromatic ring was replaced by an alkyl moiety, but which still contain the acrolein group, proved also to be active inhibitors (Brackman *et al*. 2011).

In general, the inhibitory effect of cinnamaldehyde analogs is highly dependent on the nature and degree of substitution of the aromatic ring, and the substituents with electron-withdrawing properties increase its activity. The CN and their analogs furthermore proved to be active blockers of virulence *in vivo* in different models, suggesting that they may have potential for therapeutic applications in humans and animals (Brackman *et al*. 2011).

The CN also has inhibitory activity on biofilm formation in a methicillin-resistant *Staphylococcus aureus* at dose-dependent manner and represses the expression of *sarA*, a gene implicated in the regulation of its biofilm (Jia *et al*. 2011). In *Streptococcus pyogenes*, when the biofilm was treated with CN and their derivatives the *2-nitro-cinnamaldehyde* (Figure 3.3B), *4N-*

4-nitrocinnamaldehyde (Figure 3.3G) and *4D-4-dimethylaminocinnamaldehyde* (Figure 3.3H), the biomass, average thickness and colony size at substratum were decreased and the molecular docking shows sequence and structure similarity with the active site for QS inhibition (Beema *et al*. 2014).

Among the cinnamaldehyde-related molecules, the *caffeic acid* (CA) (Figure 3I) and *ferulic acid* (FA) (Figure 3.3J) have shown antibiofilm properties. CA is the first phenolic acid compound that has been reported to have inhibitory activity on biofilm formation in *Staphylococcus epidermis* by a mechanism that did not involve bacterial death (Zimmer *et al*. 2014). The potential of FA to control biofilm formation has been demonstrated by the reduction in mass and metabolic activity in *Escherichia coli* and *Listeria monocytogenes* biofilms, and also this compound caused the total inhibition of motility in both bacteria and the colony spreading in *S. aureus*; a form of passive bacterial movement was also inhibited (Borges *et al*. 2012).

The QS inhibitory activity of CA and FA also was evaluated in *C. violaceum*, and the results revealed that the activity was mediated by their ability to modulate AHL activity and synthesis (Borges *et al*. 2013). Other related compound the *p-coumaric acid* (Figure 3.3K) showed QS inhibition in reporter strains like *C. violaceum*, *Agrobacterium tumefaciens* and *Pseudomonas chlororaphis* (Bodini *et al*. 2009).

In addition, it represses the expression of regulatory genes of the T3SS of the phytopathogenic bacteria *Dickeya dadantti*, and for this activity, its hydroxyl group on the phenyl ring and the double bond are important (Li *et al*. 2009). Some of their derivatives such as *TS027* (Figure 3.3L) and *TS110* (Figure 3.3M) also repress the expression of T3SS regulatory genes and inhibit T3 effector protein in *P. aeruginosa* without affecting its growth (Yamazaki *et al*. 2012). While the *cinnamic acid* and *4-methoxy-cinnamic acid* (Figure 3N) suppress the expression of

T3SS in *Erwinia amylovora* (Khokhani *et al*. 2013), the *o-coumaric acid* (isomer of 3.3M) and *trans-2-methoxy-cinnamic acid* (Figure 3.3O) suppress translocation of two effector proteins of T3SS in *Xanthomonas oryzae* (Fan *et al*. 2016).

3.3.3. Coumarin and related compounds

The coumarins are compounds that have caused great interest for their pharmacological properties such as anti-inflammatory, antitumor, antioxidant and bactericidal activity (Jain & Joshi 2012).

Moreover, recently it has also documented that they possess anti-virulence properties. The *coumarin* (Figure 3.4A) and *umbelliferone* (Figure 3.4B) inhibit biofilm formation of *E. coli*, without affecting its growth. By a transcriptional analysis, it was identified that these phenols act by repressing genes related to curli production and motility, which causes a decrease in the production of fimbriae and swarming (Lee *et al*. 2014). For these molecules, the hydroxylation of coumarin is an important determinant for their antibiofilm activity, since the position of hydroxyl groups as well as their number affects the antibiofilm compound activity (Lee *et al*. 2014).

Similarly, the presence of characteristic functional groups promotes the effective inhibition of virulence factors, as in the case of the furocoumarins (Girennavar *et al*. 2008), *dihydroxybergamottin* (Figure 3.4C) and *bergamottin* (Figure 3.4D), which exhibit anti-quorum-sensing effect on the AI-1 and AI-2 systems in *Vibrio harveyi*.

Similarly, these furocoumarins inhibit biofilm formation of *E. coli, V. harveyi, Salmonella typhimurium* and *P. aeruginosa* without affecting bacterial growth. Although their mechanism of action is unknown, it is suggested that the presence of a furan residue could be acting as a competitive inhibitor for binding with the receptor protein of natural bacterial autoinducers (Girennavar *et al*. 2008).

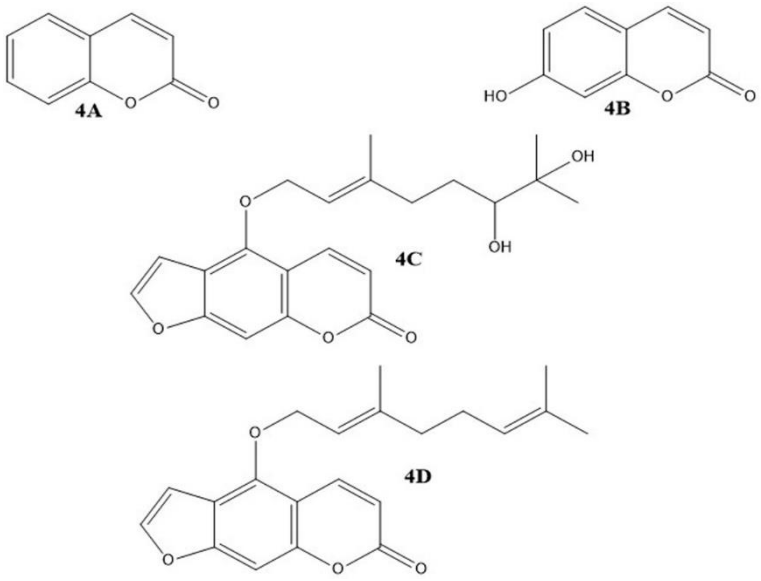

Figure 3.4. Coumarin and related compounds with anti-virulence properties. A: Coumarin, B: umbelliferone, C: dihydroxybergamottin, D: bergamottin.

3.3.4. Curcumin and related compounds

The major constituent of turmeric (*Curcuma longa* L.) roots/rhizomes is the *curcumin* (CUR) (Figure 3.5A), which is an active compound that showed an important antimicrobial activity (Aggarwal *et al*. 2007; Araújo & Leon 2001), but several studies also corroborate their inhibitory activity against virulence factors in pathogenic bacteria.

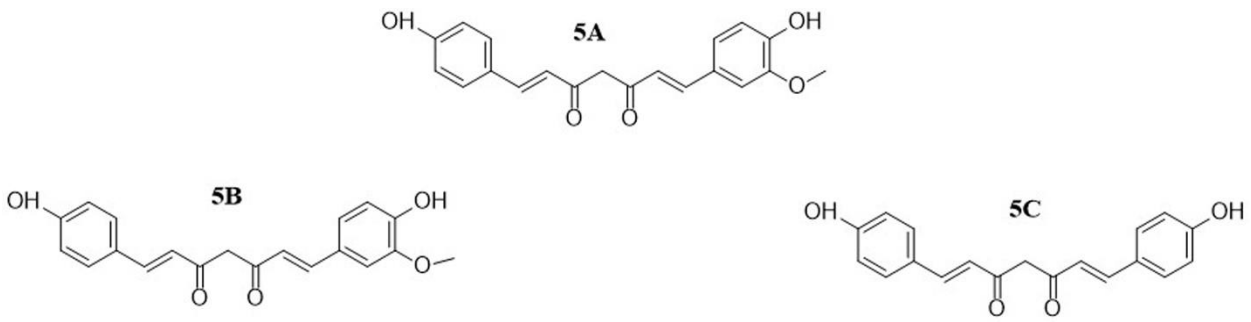

Figure 3.5. Curcumin and related compounds with anti-virulence properties. A: Curcumin, B: demethoxycurcumin, C: bisdemethoxycurcumin.

The secretion of sortase A (SrtA) a surface protein in *S. aureus* involved in bacterial adhesion for pathogenesis was inhibited by CUR, and also on *in vivo* assays, this compound reduces the capacity of bacteria to adhere to surfaces in a dose-dependent manner (Park *et al*. 2005). The other derivatives present in turmeric extract are *demethoxycurcumin* (Figure 3.5B) and *bisdemethoxycurcumin* (Figure 3.5C), which show inhibitory activity of SrtA (Park *et al*. 2005).

Similarly, in *Streptococcus mutans* CUR inhibited the activity of SrtA and other proteins implicated in bacterial adhesion reducing the biofilm formation in this bacteria (Hu *et al*. 2013a, 2013b). The diverse biological properties of CUR and its derivatives are attributed to the hydroxyl and phenol groups in the molecule (Jayaprakasha *et al*. 2006), and structure-activity relationship studies suggest that a hydroxy group at the para-position is most critical for the expression of biological activity in these compounds (Kim & Kim 2001).

The antibiofilm activity of CUR against uropathogens such as *E. coli, Proteus mirabilis* and *Serratia marcescens* was evaluated, and the results showed that their biofilm maturation was disturbed by a biomass reduction and by the interruption of swimming motility (Packiavathy *et al*. 2014). In clinical isolates of *Klebsiella pneumoniae*, the treatment with CUR was also effective for biofilm inhibition (Magesh *et al*. 2013) as well in enterohemorrhagic *E. coli* (Lee *et al*. 2011).

In the same way, in *Vibrio* spp. the inhibitory effect on biofilm formation with the CUR treatment depends on the disruption of the maturation of biofilms and in the reduction of swimming and swarming motility. Further, this compound significantly represses other virulence factors like alginate and exopolysaccharide production and also inhibits bioluminescence. These inhibitory effects were also demonstrated on *in vivo* models in which CUR enhanced the survival rate of *Artemia nauplii* against *Vibrio harveyi* (Packiavathy *et al*. 2013).

Diverse virulence factors in *P. aeruginosa* were inhibited by CUR, specifically the elastase, protease and pyocyanin production without affecting bacterial growth in a dose-dependent manner. The biofilm inhibition effect was demonstrated *in vivo* using *Arabidopsis thaliana*, where the treatment with CUR caused a reduction in the plant mortality by suppressing biofilm formation (Rudrappa & Bais 2008). In the pathogenicity model using *Caenorhabditis elegans*, CUR demonstrate their anti-infective properties by reducing the nematode mortality (Rudrappa & Bais 2008). Additionally, in *P. aeruginosa* and *C. violaceum*, CUR showed an anti-quorum sensing activity by inhibiting the production of acyl homoserine lactones (Rudrappa & Bais 2008).

3.3.5. Eugenol and related compounds

Eugenol (EG) (Figure 3.6A) is a major component of clove oil that possesses various biological properties (Qiu *et al*. 2010), and their anti-virulence activity also has been evaluated. In pathogenic bacteria that secreted a broad spectrum of virulence factors that contribute to their pathogenicity, EG showed inhibitory activity. For example, in the nosocomial pathogen *S. aureus*, the hemolysin, staphyloxanthin, toxic shock syndrome toxin 1 (TSST-1) and enterotoxins are the most important virulence factors that were remarkably affected by EG (Qiu *et al*. 2010). The expression of virulence-related genes (*sea*, *seb*, *tst* and *hla*) was also decreased after the treatment with this compound (Qiu *et al*. 2010).

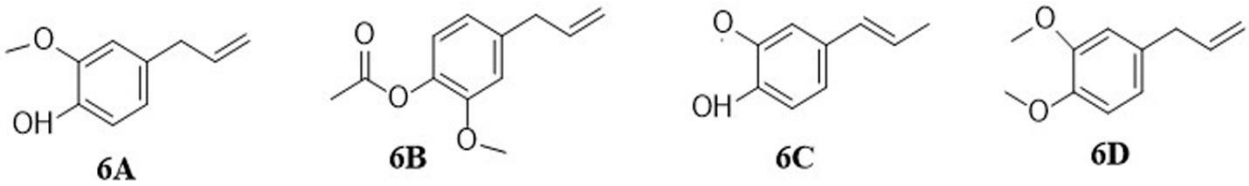

Figure 3. 6. Eugenol and related compounds with anti-virulence properties. A: Eugenol, B: eugenyl acetate, C: isoeugenol, D: methyl eugenol.

In a methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *S. aureus* at subinhibitory concentration, EG eradicates pre-established biofilms and inhibited the colonization of this bacteria in a rat middle ear model, decreasing biofilm in biomass, cell viability and the expression of biofilm-related genes (*icaD, sarA* and *seA*), resulting in a low accumulation of polysaccharides and poorly adhesion of cells within biofilms (Yadav *et al*. 2015).

The biofilm eradication effect of EG was mediated by two mechanisms: bacterial lysis within biofilms and by the disruption of cell-to-cell connections, hence dismantling the biofilm organization, which can be attributed to the hydrophobic and the lipophilic nature of their chemical structure (Yadav *et al*. 2015).

The biofilm formation and biofilm-related genes in *L. monocytogenes* and *E. coli* also were inhibited by EG at dose-dependent manner (Sikkema *et al*. 1994; Truchado *et al*. 2012; Upadhyay *et al*. 2013; Kim *et al*. 2015). In *P. aeruginosa,* although EG was unable to inhibit biofilm formation, it markedly reduced the production of pyocyanin, fimbriae production, hemolytic activity and other QS-controlled virulence factors in this bacterium such as the *Pseudomonas* quinolone signal (PQS) (Kim *et al*. 2015). Other study showed that EG at subinhibitory concentrations has QS inhibitory activity in *P. aeruginosa* and *C. violaceum* (Zhou *et al*. 2013).

Moreover, derivatives of EG *eugenyl acetate* (EA) (Figure 3.6B), *isoeugenol* (IE) (Figure 3.6C) and *methyl eugenol* (ME) (Figure 3.6D) showed anti-virulence properties against pathogenic bacteria. In *S. aureus*, EA inhibited the production of virulence factors like hemolysin and staphyloxanthin. Similarly, in *P. aeruginosa* the pyocyanin, pyoverdin and exoprotease production were significantly reduced after the treatment with EA, and it also exhibited QS inhibitory potential in *C. violaceum* (Musthafa & Voravuthikunchai 2015).

The other derivatives, IE and ME, also presented QS inhibitory against *P. aeruginosa* and *C. violaceum* (Packiavathy *et al*. 2012; Ahmad *et al*. 2015), and in the case of *V. harveyi*, ME have anti-bioluminescence activity (Packiavathy *et al*. 2012). These anti-virulence properties can be attributable to the presence of numerous substituted aromatic molecules like in the case of other phenols (Qiu *et al.* 2010).

3.3.6. Long-chain phenols

Long-chain phenols are a group of metabolites which have extensively studied antitumor, antimicrobial and antioxidant activities; they are also of great interest to the industry because they are used to manufacture different chemicals (Hemshekhar *et al*. 2012). Also, different long-chain phenols reported have different anti-virulence properties.

Our research group identified a mixture of four anacardic acids (AA) capable of inhibiting QS in *C. violaceum* and also able to reduce the production of virulence factors such as pyocyanin, rhamnolipids and elastase activity in *P. aeruginosa* (Castillo-Juárez *et al*. 2013). Similarly, another mixture of AA (Figure 3.7A) and one of cardanols (Figure 3.7B) was capable of inhibiting *P. aeruginosa* biofilms. Notably, although the antibiofilm mechanism is not known, the polymerization of the AA (Figure 3.7C) slightly potentiates the activity (Jagani *et al*. 2009).

Similarly, the maximum antibiofilm activity observed for this phenol was around 80% inhibition, which is reduced to 50% by the presence of a carboxyl group (salicylic acid) and only increases with the addition of an alkyl chain (Jagani *et al*. 2009). Hence, the incorporation of different types of alkyl chain in the meta-position of the salicylic acid seems to play a role in its activity, but this needs to be investigated in more detail.

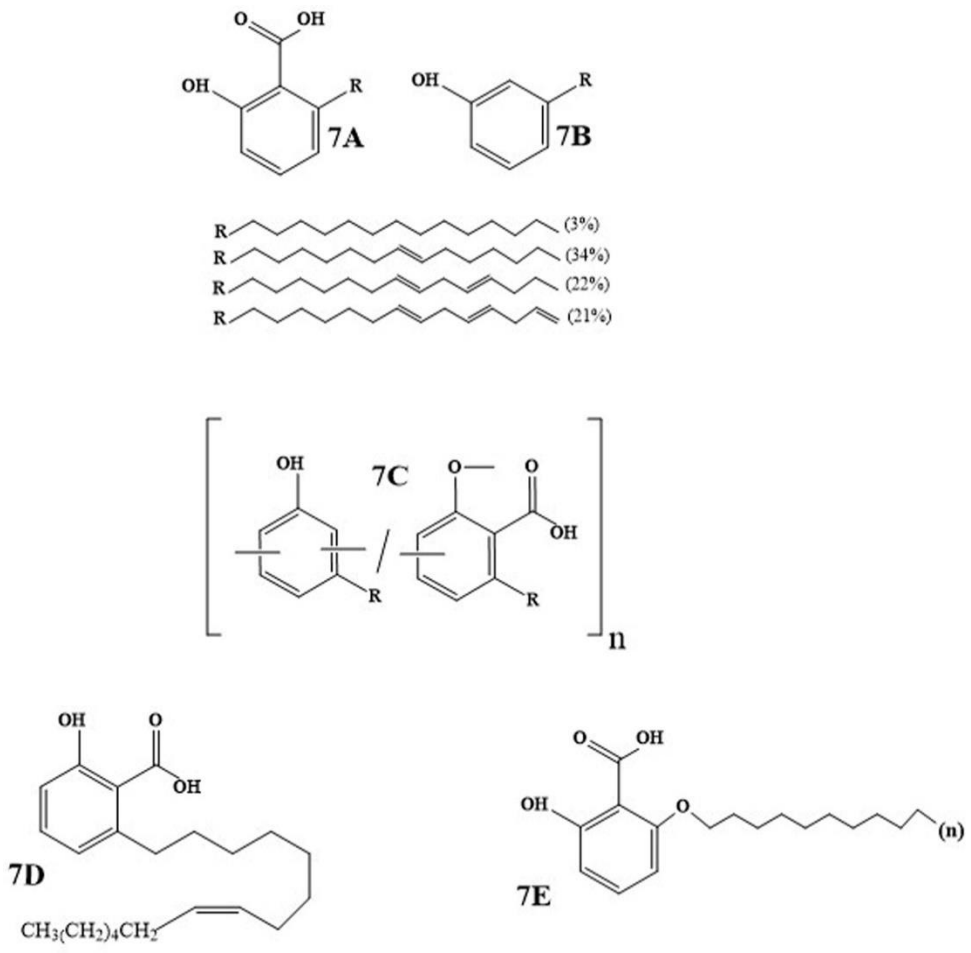

Figure 3.7. Long-chain phenols with anti-virulence properties. A: Anacardic acid mixture, B: Cardanol mixture, C: polyanacardic acid, D: ginkgolic acids C15:1, E: 6-oxa isosteres of anacardic acids.

Similarly, the antibiofilm activity of gingolic acids was reported, specifically the C15:1 (Figure 3.7D) abolished biofilm production without affecting bacterial viability, as well as reduced fimbriae production in enterohemorrhagic *E. coli* (Lee *et al*. 2014). Transcriptomic analysis by DNA microarrays and qRT-PCR demonstrated that C15: 1 represses expression of genes involved in the synthesis of curli (Lee *et al*. 2014).

Furthermore, although mixtures of such compounds have shown anti-virulence activity, separation is laborious and costly, so their chemical syntheses become an attractive alternative. In

this regard, AA synthetic (6-oxa isosteres) C: 11-C: 16 (Figure 3.7E) showed inhibition of TCS (KinA/SpoOF and NRII/NRI) (Kanojia *et al*. 1999). Interestingly, AA with alkyl chains outside this range are not active (Kanojia *et al*. 1999). Likewise, for this activity, the presence of the carboxyl group is important, as the C:12 and C:14 completely lose their effect, and the presence of phenolic OH partially restores it. Long- chain phenols are a group of natural products with great structural diversity, which represent an important potential source of molecules with anti-virulence activity.

3.3.7. Quercetin and related compounds

Various biological activities including anti-cancer, antibacterial, hepatoprotective, anti-inflammatory and antiviral activities have been attributed to flavonoids (Kumar & Pandey 2013); moreover, recent studies have shown that various flavonoids also have anti-virulence activity.

Flavonoids like *flavone* (Figure 3.8A), *quercetin* (Figure 3.8B), *apigenin* (Figure 3.8C) and *fisetin* (Figure 3.8D) decrease blood hemolysis induced by *S. aureus*. Specifically, for flavone it was elucidated that its activity is due the repression of the transcription of α-hemolysin genes (*hla*) and the global regulator gene (*Sae*) (Lee *et al*. 2012).

In addition, antibiofilm activity in *S. aureus* by quercetin (Figure 3.8B), *chrysin* (Figure 8E), *apigenin* (Figure 3.8C), *kaempferol* (Figure 3.8F) and *fisetin* (Figure 3.8D) has been reported where the number of hydroxyls is directly related to the increase in the activity (Cho *et al*. 2015), whereas *morin* (Figure 3.8G), *myricetin* (Figure 3.8H), *quercetin* (Figure 3.8B) and *kaempferol* (Figure 8F), having a hydroxyl group at C-2´ and C-4´ in ring B, inhibit SrtA and SrtB sortases of *S. aureus* more effectively (Kang *et al*. 2006).

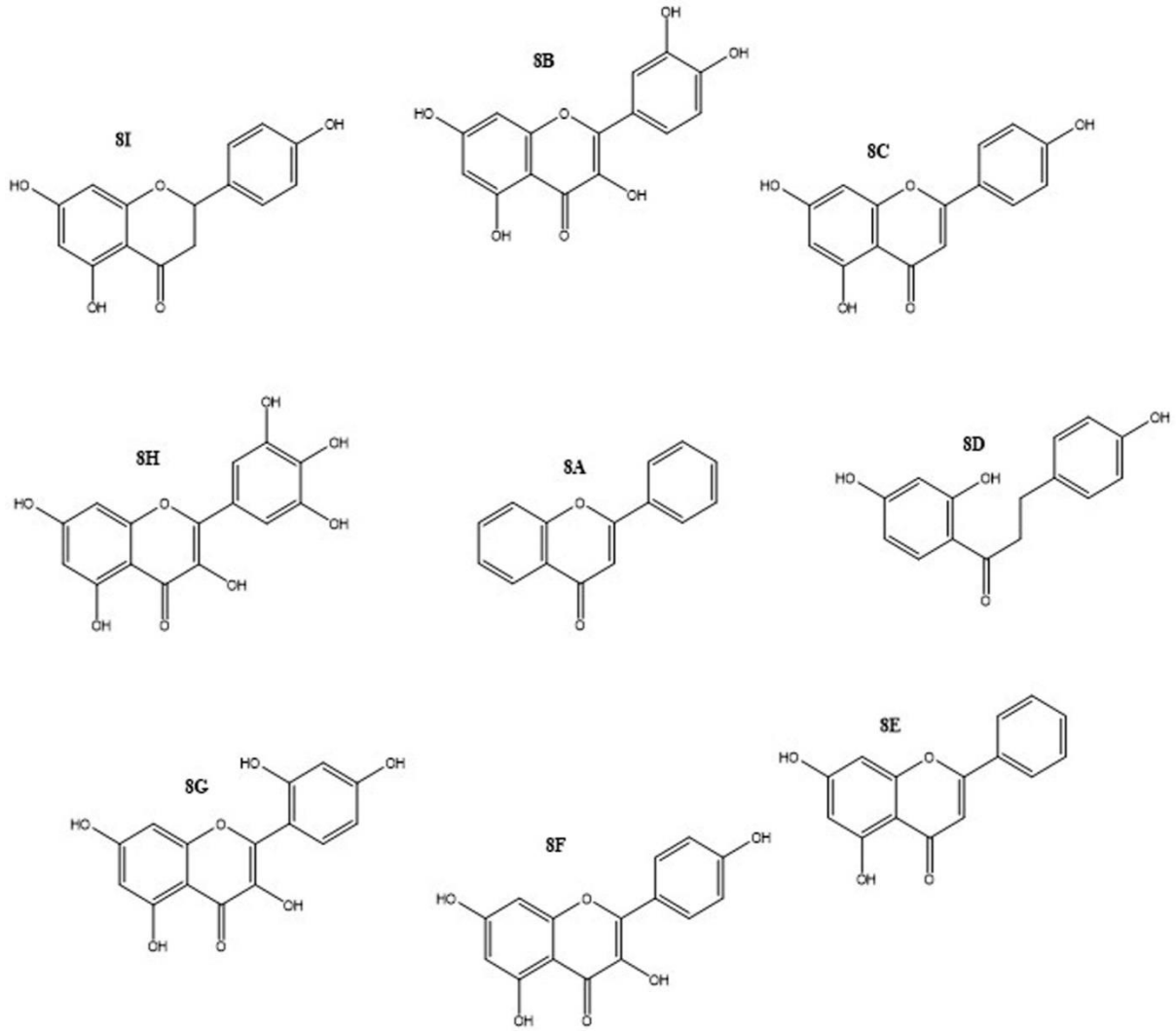

Figure 3. 8. Quercetin and related compounds with anti-virulence properties. A: Flavone, B: quercetin, C: apigenin, D: fisetin, E: chrysin, F: kaempferol, G: morin, H: myricetin, I: naringenin.

The *myricetin* (Figure 3.8H) is a compound able to interact with listeriolysin O, a virulence factor of *Listeria monocytogenes* that is involved in the lysis of host cells. This interaction is related to the presence of the double bond in the molecule, specifically in the C1-C2 position in ring C (Wang *et al*. 2015). This generates a complex that blocks the hemolytic activity of the listeriolysin as it prevents binding to cholesterol.

Furthermore, it has been shown that the naringenin (Figure 3.8I) have antibiofilm activity on *V. harveyi* and *E. coli*; however, this activity is compromised when sugar residues are incorporated (Vikram *et al*. 2010). In the case of *V. harveyi*, the *naringenin* also represses the expression of T3SS regulatory genes (Vikram *et al*. 2010).

3.3.8. Resveratrol and related compounds

Resveratrol(RV) (Figure 3.9A) is a natural polyphenol and phytoalexin produced by plants in case of attacks by pathogens (Wang *et al*. 2006). It is mainly found in the skin of grapes, some berries and red wine (Augustine *et al*. 2014). For its medical properties, it is recognized as a compound that provides multiple benefits to human health (Baur & Sinclair 2006) and recent studies have demonstrated its anti-virulence potential.

Since plants produce RV, this metabolite was identified as the active compound with inhibitory activity against biofilm formation in *Propionibacterium acnes* from extracts of plants used in traditional Chinese medicine (Coenye *et al*. 2012). Also in *S. aureus*, the evaluation of different commercial red wines showed a dose-dependent inhibition of biofilm formation, hemolytic activity and increase in the survival of *Caenorhabditis elegans* exposed to the bacteria (Cho *et al*. 2015). One of the major constituents of these red wines was RV, and similarly, it inhibited hemolysis in *S. aureus* (Cho *et al*. 2015).

In *Vibrio cholerae*, the biofilm formation has a prominent role in pathogenesis and RV was found to be a potent biofilm inhibitor at subinhibitory concentrations and showed binding affinity with the virulence activator AphB (Augustine *et al*. 2014). Furthermore, in the uropathogenic bacteria, *Proteus mirabilis* RV inhibited swarming motility, hemolysin and urease activity as well as the virulence factor expression at dose-dependent manner (Wang *et al*., 2006).

Compounds related to RV, the *oxyresveratrol* (Figure 3.9B), *dicinnamyl* (Figure 3.9C), *cis-stilbene* (Figure 3.9D) and *trans-stilbene* (Figure 3.9E) also were evaluated against *S. aureus* virulence. Only, the *cis-stilbene* and *trans-stilbene* along with RV markedly inhibited the hemolytic activity by more than 80%, while *dicinnamyl*, *oxyresveratrol* and *trans-stilbene* have a significant biofilm inhibition effect (Lee *et al*. 2014). The inhibitory activity of *trans-stilbene* was corroborated with the evidence that is able to repress the expression of the α-hemolysin gene (*hla*) and of genes implicated in adhesion (*icaA* and *icaD*) and with the attenuation of *S. aureus* virulence in the nematode *C. elegans* (Lee *et al*. 2014).

In enterohemorrhagic *E. coli*, the RV isolated from the extract of *Carex dimorpholepis* significantly reduced biofilm formation (up to 90%), expression of biofilm related genes and swimming and swarming motilities, suggesting that this compound is a major antibiofilm component in this extract, corroborating its potential as therapeutic agent against *E. coli* (Lee *et al*. 2013).

The RV and its oligomers, *namely ε-viniferin* (Figure 3.9F), *suffruticosol A* (Figure 3.9G), *suffruticosol B* (Figure 3.9H), *vitisin A* (Figure3. 9I) and *vitisin B* (Figure 3.9J) isolated from different plant families, also have antibiofilm activities against *E. coli*. The qRT-PCR analyses showed that *ε-viniferin*, *suffruticosol B* and *vitisin B* repress the expression of genes involved in curli and fimbriae production (Lee *et al*. 2014) Also, RV and *suffruticosol A*, *suffruticosol B*, *vitisin A* and *B* inhibit biofilm formation in *P. aeruginosa* at dose-dependent manner (Lee *et al*. 2013).

The oligomers *ε-viniferin* and *trans-gnetin* (Figure 3.9K) isolated from *Paeonia lactiflora* have inhibitory activity in neuraminidase activity, an enzyme involved in many pathological process in tropical human pathogens (Yuk *et al*. 2013). Furthermore, the *ε-viniferin* and RV

isolated from *Carex pumila* extract also demonstrated significantly biofilm inhibition in *P. aeruginosa* and *E. coli* (Cho *et al*. 2013).

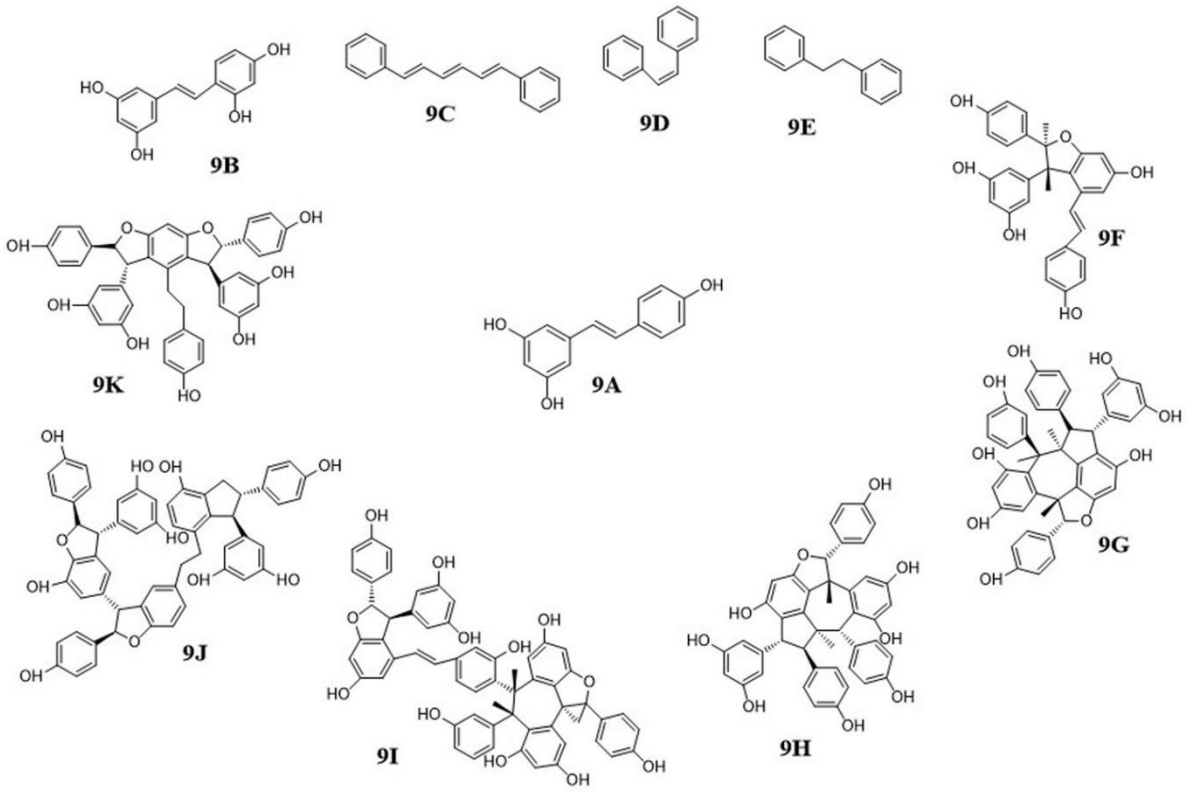

Figure 3. 9. Resveratrol and related compounds with anti-virulence properties. A: Resveratrol, B: oxyresveratrol, C: dicinnamyl, D: cis-stilbene, E: trans-stilbene, F: ε-viniferin, G: suffruticosol A, H: suffruticosol B, I: vitisin A, J: vitisin B, and K: trans-gnetin.

The anti-quorum sensing activity of RV also was demonstrated, in *C. violaceum*, since it reduces violacein production (Alvarez *et al*. 2012; Truchado *et al*. 2012). In *Yersinia enterolitica* and *Erwinia amylovora*, it was one of the most active compounds that can reduce the concentration of the autoinducers due to degradation transformation or inhibition of synthesis (Truchado *et al*. 2012).

3.3.9. *Salicylic acid and related compounds*

Salicylic acid (SA) (Figure 3.10A) is a phenolic compound synthesized by plants that play an important role in the regulation of various physiological processes (Prithiviraj *et al*. 2005; Vlot *et al*. 2009), and in recent years, their inhibitory activity against bacterial virulence has been reported.

Several studies have demonstrated that SA has inhibitory activity in the motility and production of extracellular virulence factors in the opportunistic pathogenic bacteria *P. aeruginosa*, and among those factors, pyocyanin was inhibited by approximately 80% by SA and decreased the elastase and exoprotease production (Prithiviraj *et al*. 2005). Similarly, a subinhibitory concentration of SA inhibited the twitching and swimming motility as well as the invasion and acute cytotoxicity of *P. aeruginosa* in corneal epithelial cells (Bandara *et al*. 2006).

Some derivatives of SA, including *acetyl salicylic acid* (Figure 3.10B), *salicylamide* (Figure 3.10C), *methyl salicylate* (Figure 3.10D) and a precursor of SA, *benzoic acid* (Figure 3.10E), were evaluated, and the inhibition levels observed were comparable with those obtained with SA for the same virulence factors (Prithiviraj *et al*, 2005). SA is a *benzoic acid* that possesses an aromatic ring bearing a hydroxyl group, and probably, one of these components of the structure is responsible for its anti-virulence activity.

The biofilm formation in *P. aeruginosa* was also inhibited by SA *in vitro* and *in vivo* decreasing the attachment and consequently the biofilm formation (Jagani *et al*, 2009; Prithiviraj *et al*. 2005). Similarly, in other bacterial pathogenic species that form biofilms, SA has inhibitory activity; for example, in *Streptococus mutans*, the biofilm formation was highly decreased when the enzymes, glucosyl and fructosyl transferases, which synthetize extracellular polymeric substances, were inhibited by SA (Sendamangalam *et al*. 2011).

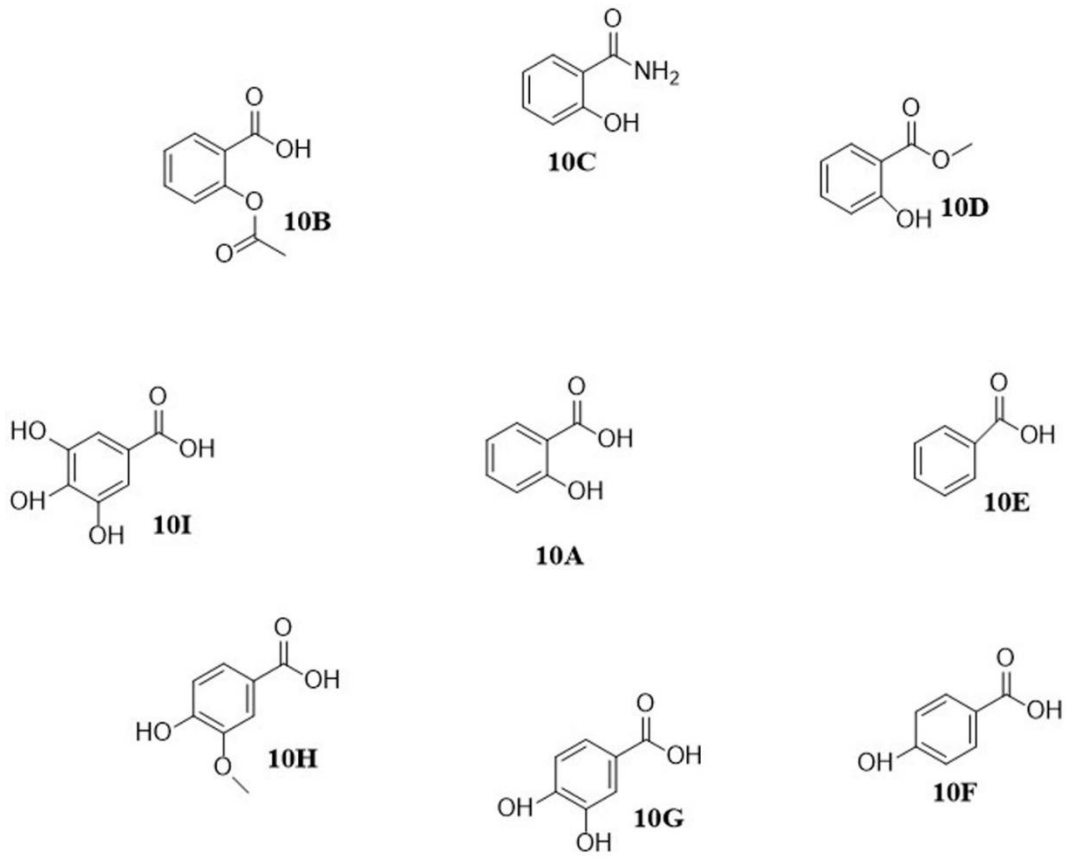

Figure 3.10. Salicylic acid and related compounds with anti-virulence properties. A: Salicylic acid, B: acetyl salicylic acid, C: salicylamide, D: methyl salicylate, E: benzoic acid, F: p-hydroxybenzoic acid, G: protocatechuic acid, H: vanillic acid, I: gallic acid.

Compounds related to SA, the *p-hydroxybenzoic acid* (Figure 3.10F) and *protocatechuic acid* (Figure 3.10G) at growth subinhibitory concentrations have different modes of action on biofilm formation disruption in *Staphylococcus* species (Morán *et al*. 2014). Also, for the bacteria *Helicobacter pylori* implicated in the development of peptic ulcer, duodenal ulcer and gastric cancer, which uses a urease enzyme for the basification of the stomach pH and hence the colonization of the gastric mucosa (Benoit & Maier 2003), the *protocatechuic acid* has an inhibitory effect of 40% in its urease activity (Liu *et al*. 2008).

Vanillic acid (4-hydroxy-3-methoxybenzaldehyde) (Figure 3.10H) also showed antibiofilm activity in *Aeromonas hydrophila* at all the concentrations used in the range of 0–0.250 mg/mL (Ponnusamy *et al*. 2009). Other important hydroxy benzoic acid with a numerous reports of anti-virulence properties is *gallic acid* (GA) (Figure 3.10I), which shows inhibition in many virulence factors among bacteria.

For example, in *S. aureus*, GA reduces the bacterial adhesion and biofilm formation as well as the production of α-hemolysin a virulence factor produced by the bacteria with hemolytic, cytotoxic, dermonecrotic and lethal properties (Bhakdi & Tranum-Jensen 1991) since its activity was inhibited in a dose-dependent manner by this compound (Luís *et al*. 2014).

Similarly, in *P. aeruginosa*, *E. coli* and *Listeria monocytogenes*, their biofilm formation was also inhibited by GA. The inhibitory activity showed by these compounds may be related to some of their structural features, since different reports mentioned that in the active phenolic compounds, the basic skeleton remains the same, the basic skeleton remains same, but the number and positions of the hydroxyl groups on the aromatic ring and the type of substituents provide different biological properties (Robbins 2003; Sroka & Cisowski 2003; Stalikas 2007).

Also, *SA*, *gallic acid* and *vanillic* acid have QS inhibitory activity by two different mechanisms: first, by affecting the synthesis of AHLs (Plyuta *et al*. 2013; Truchado *et al*. 2012; Chang *et al*. 2014) and second, by interfering with the binding of short-chain AHLs to their receptor, especially in the case of vanillic acid (Ponnusamy *et al*. 2009).

3.4. Conclusion and future perspective

An important feature of the anti-virulence molecules is that they may be less prone to promote the emergence of resistance than conventional antibiotics. At the moment, phenolic compounds

represent the largest number of natural products with anti-virulence-reported activity and whose main target has been the inhibition of QS and biofilms.

However, it has also been found that they can directly inhibit some of virulence factors such as sortases, curli, type III secretion system (T3SS), fimbriaes and two-component regulatory systems. It should be noted that most of the phenolic compounds represent structures already known, several of which have been subject to different pharmacological studies and some are even part of the international pharmacopeia and are active ingredients of herbal medicines.

Moreover, although QS is considered the main regulator of bacterial virulence, this is still part of a complex network of interconnected components including several environmental regulation systems and QS-independent virulence factors. Also, the direct inhibition of virulence factors and regulators of QS and TCS represents interesting options for achieving the implementation of this strategy. Thus, the correct design of anti-virulence therapies is very important (García-Contreras 2016; Weigert *et al*. 2016), and a feasible option is the combination of drugs with different action targets.

In the same way, some challenges to overcome involve the evaluation of anti-virulence compounds in most bacterial systems, the corroboration in vivo in animal infection models and finally the evaluation of possible side effects on the populations of commensal and symbiotic bacteria.

Given the growing public health problem worldwide derived by the emergence of bacterial multiresistance to antibiotics, the development of suitable anti-virulence therapies is presented as a viable strategy to provide a solution to this problem; moreover, we are in the decisive years that will dictate the implementation of these kind of strategies, this is occurring in a period

of resurgence of the interest in natural products activities in which phenolic compounds have a fundamental role.

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CHAPTER IV. NATURAL PRODUCTS WITH QUORUM QUENCHING INDEPENDENT ANTI-VIRULENCE PROPERTIES

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Natural products with quorum quenching independent anti-virulence properties

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4.1. Abstract

Resistance to antibiotics is becoming a major global threat to the effective combat of infections caused by bacteria. Bacterial strains resistant to multiple antibiotics is becoming more common, particularly in hospital centers, where there is an environment with high selective pressure. The search for new more effective antibacterial strategies able to reduce the development of resistance has become a global challenge. The development of anti-virulence therapies has been proposed as a viable strategy to help combat antibiotic-resistant strains or at least to diminish their appearance, by not generating resistance. One of the most intensely studied anti-virulence strategies for therapy consists in the disruption of quorum sensing, a strategy also known as "quorum quenching" (QQ), consisting of disrupting bacterial cell to cell communication, mainly with natural products. To date several types of quorum sensing systems have been described in various bacterial pathogens, and others are still being discovered. Concomitantly, a plethora of compounds with the ability to inhibit

quorum sensing has also been described. Nevertheless, many virulence factors are either not regulated by QS or are negatively regulated by this system; hence, in this chapter the most recent work related to the activities of natural products with QQ-independent anti-virulence activity is discussed. Also, we emphasize that the mechanism of action of these compounds can complement QQ to develop new anti-virulence with higher success rates than strategies based solely in QQ.

Keywords: quorum quenching; anti-virulence; natural products, new antimicrobials, virulence factors.

4.2. Introduction

Multidrug resistant bacteria constitute a serious threat to human health. Inappropriate prescription of antibiotics, their indiscriminate use in agriculture and livestock industries, and the lack of patient adherence to schemes are the cause of the constant appearance of resistant strains (Doyle 2015; Ventola 2015). The situation is so dire that the World Health Organization warned that we may enter a "post-antibiotic era" within this century; accordingly, bacteria resistant to all known antibiotics are becoming common and are already producing untreatable infections (Soo *et al* 2016). Because of the increasing threat of these pathogens and their continuous evolution of resistance, there is an urgent need to develop new antibacterial therapies (Escaich, 2010).

An alternative to using antibiotics to fight bacterial infections is inhibiting virulence factors that pathogens require to cause damage (Defoirdt 2016; Ruer *et al*. 2015). This new strategy has been called anti-virulence therapy and can be used to control bacterial diseases in animals and plants (Defoirdt 2016; Ruer *et al*. 2015). The principal advantage of this strategy is that, by not interfering with the viability of the bacteria, the probability of its promoting resistance is lower (Rasko & Sperandio 2010).

One of the most intensely studied strategies is inhibition of quorum sensing (QS) (bacterial cell to cell communication) mainly by the utilization of small molecules that bind and inhibit the QS receptors, hence impairing the ability of pathogens to sense their population density, a strategy known as quorum quenching (QQ) (Defoirdt 2016). Although reports of QS inhibitors are abundant, most of the positive effects have been demonstrated only *in vitro*. Nevertheless, in animal models an increase in survival and a decrease in damage to the host have been achieved through QQ, although quorum quenchers are not always able to completely eradicate the infection (Castillo-Juarez *et al*. 2015). Indeed, recent studies demonstrated that virulence is a complex interplay between the host and pathogen factors (a multifactorial phenomenon) so that the manipulation of virulence factors production *in vivo* may produce unexpected responses (Weigert *et al*. 2016).

Moreover, although QS systems in general up-regulate the expression of virulence factors, some relevant exceptions have been found. As discussed in the following section, some bacterial secretion systems and adherence proteins such as curli and fimbriae are repressed by QS. Furthermore, some virulence factors not regulated by QS have been identified (e.g. lipase and hemolysin in *Vibrio harveyi*) (Natrah *et al*. 2011).

Hence, to develop robust anti-virulence therapies, it may be necessary to study virulent behavior *in vivo* and to determine the spatial-temporal expression of virulence factors and their role in active infections. From studies of this type, we can obtain valuable information addressing the importance of QS-activated, QS-repressed and QS-independent virulence factors. In this chapter, we focus on the importance of virulence determinants that are not positively regulated by QS as well as on current knowledge of their inhibition by several natural products.

4.3. Quorum sensing as master regulator of virulence.

Quorum sensing (QS) is a cell-to-cell communication system used by several species of pathogenic bacteria; it allows them to coordinate the expression of virulence factors when they reach a sufficiently high cell density. It is likely that evolution selected this mechanism since the expression of virulence factors is energetically costly (Diggle *et al*. 2007).

Moreover, these factors often activate the immune system, hence their expression would be beneficial only after reaching a critical bacterial mass, when they would have the opportunity to establish an infection (de Kievit & Iglewski 2000). Furthermore, their expression at low cell density could be a burden, rather than a benefit for the bacterial population. These systems function by the secretion of small diffusible signals called autoinducers, that are produced at a basal rate at low cell densities, but when the population reaches a threshold concentration, the autoinducers bind to their receptors which directly or indirectly activate the expression of several genes, including those of the enzymes in charge of the autoinducer production as well as a plethora of virulence factor genes (Figure 4.1) (Castillo-Juarez *et al*. 2015; Papenfort & Bassler 2016).

To date, although QS systems have been identified in several bacterial pathogens, only the QS systems of a few pathogenic bacteria such as *Vibrio*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been studied in detail (Jimenez *et al*. 2012; Castillo-Juarez *et al*. 2015).

Due to the relevance of QS systems as master regulators of bacterial virulence, their inhibition has been proposed as an alternative strategy to combat bacterial infections, especially now that we are facing a severe multi-antibiotic resistance crisis. QQ could be achieved by interfering with the autoinducer-receptor binding (e.g. by blocking the receptors using

nonfunctional signal analogues), by degrading autoinducer signals and by inhibiting autoinducer production (directly targeting the autoinducer producing enzymes) (Castillo-Juarez *et al*. 2015; Papenfort & Bassler 2016).

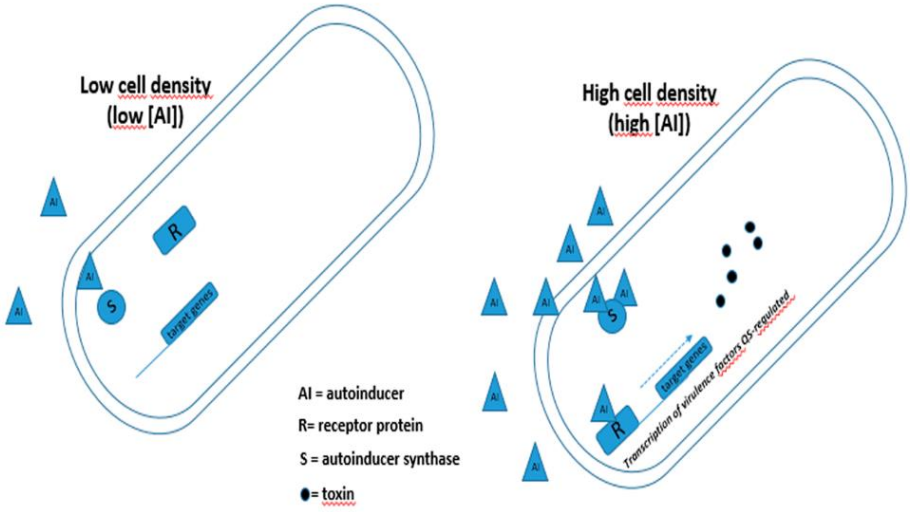

Figure 4.1. Simplified schematic of a generalized bacterial quorum sensing system.

One of the more attractive features of QQ is that since virulence, and not growth, is the target of the therapy, it has been suggested that QQ would not generate bacterial resistance. However, several experimental and theoretical studies indicate that this may not be the case, due to several factors, including the QS regulation of metabolic processes that *in vivo* are linked to growth and survival, the positive effect of QS in the response to stress, and the inherent role of QS-dependent virulence factors such as exoproteases and siderophores for bacterial fitness during infections. Moreover, several clinical isolates not sensitive to current QS inhibitors have been identified (Maeda *et al*. 2012; García-Contreras *et al*. 2013a; García-Contreras *et al*. 2013b; Kalia *et al*. 2014; Defoirdt 2016; García-Contreras 2016b; Pérez-Velázquez *et al*. 2016; Wei *et al*. 2016).

4.4. Some important virulence factors are downregulated by QS

The injectisome or type III secretion system (T3SS) is a multiprotein apparatus that facilitates the secretion and translocation of toxins or effector proteins from the bacterial cytoplasm directly to the eukaryotic cell (Figure 4.2) (Aiello *et al*. 2010; Gu *et al*. 2015). The fimbriae/pili are fibrous proteinaceous structures involved in the adhesion and recognition of host cells (Knight & Bouckaert, 2009) (Figure 4.2).

Recent studies have shown that in some cases the expression of these virulence determinants is negatively regulated by QS, since in some pathogenic bacteria expression of T3SS and fimbria/pili increases when QS is turned off (e.g. low density cell) and is inhibited when a high cell density is reached (Figure 4.2). Hence, if we consider that the best anti-virulence strategy is one that fully inhibits virulence, QQ in these cases would be only partially optimal, because its inhibition may favor the expression of other virulence factors (García-Contreras 2016).

In fact, it was recently documented that environmental *P. aeruginosa* strains, which are defective in QS due to mutations that inactivate the QS master regulator LasR, are more aggressive and cause more severe damage in bacterial keratitis, probably due greater expression of CupA fimbriae (Hammond *et al*. 2016).

Moreover, *in vitro* assays for identification of activity of QQ-employing reporter-strains, false positives can occur, as reporter activities are often dependent on other factors such as metabolic activity (Defoirdt 2016).

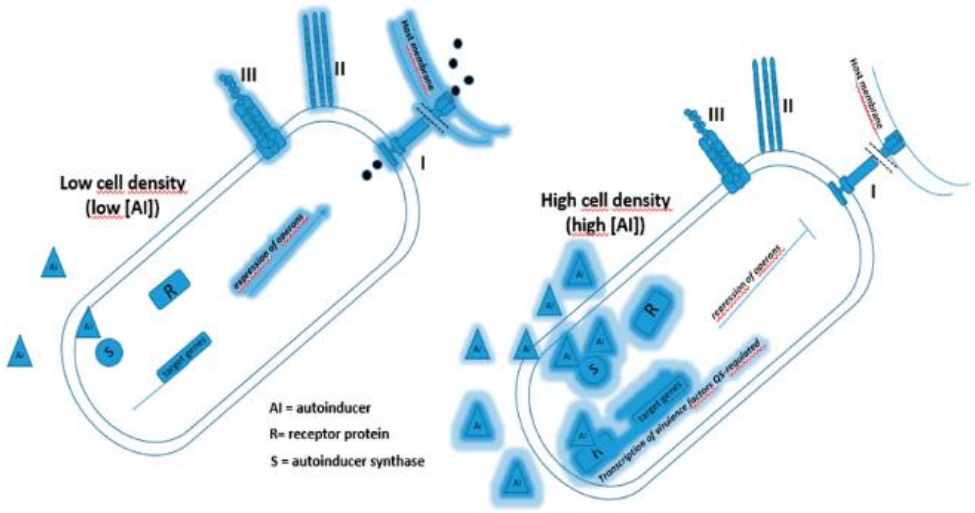

Figure 4.2. Virulence factors downregulated by QS. In some bacterial genera, QS can also downregulate the expression of other important determinants related to damage to the host, such as type III secretion system (I), fimbriae (III) and pili (III).

On the other hand, *in vivo* studies, host factors may be involved in the regulation of virulence factors, such as the presence of catecholamines (Yang *et al*. 2014), cholesterol, mucin (Li *et al*. 2015) and low phosphate (Lamarche *et al*. 2008) among other yet unidentified factors (Ruwandeepika *et al*. 2015). In this regard, it is worth mentioning that most studies of natural products with QQ activity have focused on investigating the expression and regulation of virulence factors using *in vitro* tests, where bacterial growth occurs in synthetic media cultures, usually rich in nutrients (Defoirdt *et al*. 2013).

However, growth in complex natural environments (such as hosts) is very different from growth in standard monoculture conditions (Rasko & Sperandio 2010; Ruer *et al*. 2015). Moreover, we do not yet completely understand the global regulation of virulence factors by QS *in vivo* during bacterial infections, where other regulatory mechanisms are present and not taken

in to account in available simplified *in vitro* models. Hence, understanding these mechanisms, would contribute to the development of effective anti-virulence therapies.

Chromobacterium violaceum is a saprophytic soil bacteria and an opportunistic pathogen of animals and humans (de Oca-Mejía *et al*. 2015). Although reported cases of human infection by this bacterium are considered low, relative to other bacteria of medical importance, *C. violaceum* infections have a major impact on public health due to their high mortality rate (Rai *et al*. 2011). In recent decades, the number of documented cases has increased and the geographical distribution of the microorganism is expected to expand due to the rise in temperature caused by global climate change (Yang & Li 2011).

In this bacterium, the production of the pigment violacein is positively regulated by QS, specifically by a single system based on acyl-homoserine lactones autoinducers (de Oca-Mejía *et al*. 2015; Durán *et al*. 2016). Thus, the apparent simplicity of its QS system and the easy visualization of the pigment have made *C. violaceum* a major QQ biosensor used to determine the activity of a plethora of natural products (Silva *et al*. 2016). This allows identification of a great number of natural products with QQ activity, especially those isolated from higher plants (Silva *et al*. 2016).

In addition to the production of violacein, it has been documented by studies *in vitro* that *C. violaceum* QS is also involved in the upregulation of other virulence factors such as chitinase (Chernin *et al*. 1998) and alkaline exoprotease activities (de Oca-Mejía *et al*. 2015). Also, it increases aggregation, biofilm formation, swarming, H_2O_2 stress tolerance and may influence the utilization of several carbon and nitrogen sources (de Oca-Mejía *et al*. 2015). Similarly, it controls the expression of genes that regulate the type VI secretion system (Stauff & Bassler 2011).

It has been suggested that the ecological role of violacein is an antioxidant agent and that it is involved in defense mechanisms against fungal diseases and eukaryotic predation (Becker *et al*. 2009; Konzen *et al*. 2006; Matz *et al*. 2008). Moreover, although *in vitro* studies have documented its cytotoxic activity on mammalian cells (Melo *et al*. 2000), it is not clear if it plays a role as a virulence factor.

For instance, it has been shown that a mutant of the mono-oxygenase vioA (Δ*vioA*) (a key enzyme in the synthesis of violacein) is unable to produce this pigment; nevertheless, it is able to maintain full virulent phenotype in mice model sepsis and in *Caenorhabditis elegans* infections (Swem *et al*. 2009). This demonstrates that factors other than violacein are responsible for *C. violaceum* pathogenicity. Furthermore, lifespan assays with *C. elegans* show only a delay in the death of the animals infected with a QS mutant strain Δ*cvI* (which does not produce AI synthase), compared with the wild-type strain (Swem *et al*. 2009). This suggests that other factors that are QS-independent are crucial for the overall virulence of the bacterium.

Besides violacein production, *C. violaceum* possesses genes associated with two distinct type III secretion systems (T3SSs), each coded in different pathogenicity islands (Cpi-1/-1a and Cpi-2) (Miki *et al*. 2010). It is known that the intraperitoneal infection of the bacteria induces fulminant hepatitis in mice via the T3SS encoded in Cpi-1/-1a. It was also observed that mutant strains lacking the Cpi-1/-1a-encoded T3SS do not produce death in infected mice. In contrast, a 100% mortality rate was observed when mice were inoculated with the wild-type strain. Moreover, Cpi-1/-1a-encoded T3SS is also able to produce fatal infection by induction of hepatocyte death (Miki *et al*. 2010).

Although there are no studies demonstrating the downregulation of T3SS by QS *in vivo* for *C. violaceum*, this relationship has been observed in other bacteria such as *P. aeruginosa* (Bleveset *et al*. 2005), *Aeromonas hydrophila* (Zhou & Liu 2007), *Yersinia pseudotuberculosis* (Atkinson *et al*. 2011) and *Vibrio harveyi* (Ruwandeepika *et al*. 2015).

The bacterium *V. harveyi* is a major pathogen of aquatic organisms such as shrimp and fish, as well as one of the main bacterial models for the study of QS/QQ (Defoirdt *et al*. 2007). Several virulence factors have been associated with damage caused by this bacterium, such as motility, production of lytic enzymes and siderophores (Defoirdt *et al*. 2007; Ruwandeepika *et al*. 2011), and many studies show that diverse virulence factors in *Vibrio* species are regulated by QS (Defoirdt 2014; Milton 2006). In addition, some virulence factors produced by this bacterium are translocated into host cells by T3SS (Gerlach & Hensel 2007); however, inhibition of light production has been the primary phenotype used to determine QQ activity *in vitro*.

The QS architecture of *V. harveyi* is more complex than that of *C. violaceum*, as it consists of a three-channel system regulated by three different types of AI (homoserine lactones, AI-2 and CA-I) (Durán *et al*. 2016), involving a complex regulatory cascade with cross regulation among these three systems, for which the transcriptional-regulator-protein-LuxR plays a fundamental role (Ruwandeepika *et al*. 2015).

Under conditions of high cell density, LuxR binds to its autoinducer and upregulates the *lux* operon, responsible for the production of light, and some virulence factors (Ruwandeepika *et al*. 2015). Also, it has been demonstrated that LuxR represses the expression of T3SS operons. So, in a low cell density when autoinducer production is low (inactive QS), T3SS expression is high (Henke & Bassler 2004; Ruwandeepika *et al*. 2015; Waters *et al*. 2010).

Furthermore, it is also known that during the infection process, yet unknown external factors belonging to the host appear to play an important role in the regulation of QS and T3SS in *V. harveyi* (Ruwandeepika *et al*. 2015). It is interesting that the main mechanism of action of natural products with QQ activity described so far involves interference with LuxR (Silva *et al*. 2016), although no studies to date have shown whether the expression of T3SS genes is altered by these treatments.

QS is also an important regulator of virulence in plant pathogenic bacteria, regulating processes such as motility, adhesion and the expression of enzymes that degrade the cell wall (Barnard & Salmond 2007; Cui *et al*. 2005; Põllumaa *et al*. 2012). However, although previous studies have demonstrated the central role of QS in the expression of virulence, it appears that during the infection process *in vivo*, the role of QS is more complex (Moleleki *et al*. 2016).

Pectobacterium carotovorum subsp. *brasiliense* 1692, an emerging pathogen that infects potato and causes blackleg in the field and soft rot (Nunes Leite *et al*. 2014), employs acyl-homoserine lactones as autoinducers, and interestingly, in this bacterium the expression of fimbriae and pili are negatively regulated by QS (Moleleki et al. 2016). Similarly, in *Xyllella fastidiosa* QS mediated by DSF (diffusible signal factor) downregulates type 4 pili expression (Chatterjee *et al*.2008).

There are four major categories of diarrheagenic *Escherichia coli*: enterotoxigenic, enteroinvasive, enteropathogenic and enterohemorrhagic (Levine 1987). The enterotoxigenic *E. coli* is a major cause of diarrhea in travelers and infants in less-developed countries (Levine 1987); it has been found that F4-fimbriae production is negatively regulated by AI-2 mediated QS (Zhou *et al*. 2014). In contrast, for enteropathogenic *E. coli*, it has been reported that T3SS expression is positively regulated by QS (Sperandio *et al*. 1999).

Serratia marcescens is an emerging pathogen that thrives particularly in soils in association with plants, but it is increasingly common in nosocomial infections, as well as in ocular infections in humans (Hejazi & Falkiner 1997; Labbate *et al*. 2007). Adherence to biotic and abiotic surfaces is crucial for this bacterium to colonize and cause damage to the host (Labbate *et al*. 2007). It uses an *N*-acyl homoserine lactone based QS system to regulate several processes related to surface colonization, such as swarming motility, biofilm maturation and biofilm sloughing (Eberl *et al*. 1996; Labbate *et al*. 2004).

Nevertheless, current evidence suggests that QS is only important for adhesion to abiotic surfaces, but not for adhesion to biotic surfaces such as host tissue (hydrophilic tissue culture plates and human corneal epithelial cells) and type I fimbriae has been identified as responsible for tissue binding. However, it seems that the expression of these fimbriae is not controlled by QS (Labbate *et al*. 2007). Recently, it was shown that two genes involved in fimbriae expression (*bsmA* and *bsmB*) are regulated independently of QS (Labbate *et al*. 2007), perhaps by host factors during the infection process.

4.5. Natural products as inhibitors of bacterial secretion systems and adherence

The use of natural products to inhibit QS is one of the main strategies that has been used in anti-virulence therapies. However, an alternative therapy that has also been reported is the *direct inhibition of individual virulence factors* (DIIVF), such as bacterial secretion systems, adherence molecules, toxins, two-component systems (TCS), and metabolic processes (key enzymes) involved in the formation and maturation of virulence structures such as biofilm, curli and flagella, among others (Table 4.1).

Table 4. 1. Natural products with direct inhibition of individual virulence factors

Compound	Source	Targeting factors	Targeting pathogenic bacterial strain	Pharmacological effects/target	Ref
Benzoic acid (**1**)	Several plant species	T3SS	*E. amylovora*	Reduced the hypersensitive response on tobacco leaves	Khokhani et al. 2013
4-Methoxy-cinnamic acid (**2**)	Derivate	T3SS	*E. amylovora*	Affects regulatory components of T3SS	Khokhani *et al*. 2013
o-Coumaric acid (**3**)			*X. oryze* pv. *oryzae* (*Xoo*)	Suppresses the translocation of T3 proteins	Fan *et al.* 2016
Salicylic acid (**4**)	Several plant species	T3SS	*E. amylovora*	Affects regulatory components of T3SS	Khokhani *et al*. 2013
trans-4-Mercapto-cinnamic acid (**5**)	Derivate				
trans-4-Dimethylamino-cinnamic acid (**6**)					
p-Coumaric acid **(7)**	Derivate	T3SS	*D. dadantii*	Represses the expression of T3SS genes	Li *et al*. 2009 Li *et al*. 2015
trans-4-Hydroxycinnamohydroxamic acid (**8**)					
Methyl *p*-coumarate (**9**)	Derivate	T3SS/TCS	*P. aeruginosa*	Inhibits T3SS effector protein via GacS-GacA	Yamazaki *et al.* 2012
trans-4-Amino-cinnamic acid (**10**)					
trans-2-Phenylcyclopropane-1-carboxylic acid (**11**)	Derivate	T3SS	*X. oryze* pv. *oryzae* (*Xoo*)	Suppresses the translocation of T3SS proteins	Fan *et al*. 2016
trans-2-Methoxy-cinnamic acid (**12**)					
trans-2-Methyl-cinnamic acid (**13**)					
Chalconaringenin (**14**)	*Solanum lycopersicum*	T3SS	*P. syringae pv. tomato*	Unknown	Vargas *et al*. 2013
Rutin (**15**)					
Phloretin (**16**)	*Malus pumila*	Adherence	Enterohemorrhagic *E. coli* O157:H7	Inhibits the production of fimbriae	Lee *et al*. 2011a
Quercetin (**17**)	*Solanum lycopersicum*	T3SS	*P. syringae pv. tomato*	Unknown	Vargas *et al*. 2013
	Scutellaria baicalensis		*S. enterica* serovar Typhimurium.	Inhibits T3SS by blocking synthesis and	Collazo & Galán 1997

				assembly of SP1-1	
	Bark of *Rhus verniciflua*	Toxins/ Adherence	*S. aureus*	Inhibitory effect on sortase activity (SrtA and SrtB)	Kang *et al*. 2006
(-)-Hopeaphenol (**18**)	*Anisoptera thurifera* and *A. polyandra*	T3SS	*Y. pseudotuberculosis* *P. aeruginosa*	Inhibits secretion and expression of T3SS effector proteins	Zetterström *et al*. 2013)
Baicalein (**19**)	*Scutellaria baicalensis*	T3SS	*S. enterica* serovar Typhimurium.	Blocks synthesis and assembly of SP1	Tsou *et al*. 2016
Guadinomines A (**20**) Guadinomines B (**21**)	*Streptomyces* sp. K01-0509	T3SS	Enteropathogenic *E. coli*	Unknown	Iwatsuki *et al*, 2008
Aurodox (**22**)	*Streptomyces* sp. K06-0806 and K07-0034	T3SS	*C. rodentium*	Inhibitory activity on T3SS secretion proteins. Its administration increased survival of mice	Kimura *et al*. 2011
Factumycin (**23**)				Inhibitory activity on T3SS secretion proteins	Kimura *et al*. 2011
Caminoside A (**24**), B (**25**), C (**26**) and D (**27**)	*Caminus sphaeroconia*	T3SS	*E. coli*	Inhibitory activity on T3SS secretion proteins	Linington *et al*. 2006; Linington *et al*. 2002
Pseudoceramide B (**28**) Spermatinamine (**29**)	*Pseudoceratina* sp.	T3SS	*Y. pseudotuberculosis*	Inhibits T3 protein secretion	Yin *et al*. 2011
Ginkgolic acids C15:1 (**30**) Ginkgolic acids C17:1 **(31)**	*Ginkgo biloba*	Adherence	Enterohemorrhagic *E. coli* O157:H7	Inhibits the production of fimbriae	Lee *et al*. 2014a
Coumarin **(32)** Umbelliferone **(33)** Cinnamaldehyde **(34)** Eugenol **(35)**	Several plant species Cinnamon bark oil	Adherence	Enterohemorrhagic *E. coli* O157:H7	Represses the expression of *csgA* and *csgB* genes involved in the synthesis of curli	Kim *et al*. 2016; Lee *et al*. 2014
Asiatic acid (**36**)	*Centella asiatica*	Adherence		Inhibitory activity against	

Ursolic acid (**37**)	Several plant species		Uropathogenic *E. coli*	*csgA* gene involved in synthesis of curli	Wojnicz *et al*, 2013
Berberine (**38**)	Root and bark of several plant species	Adherence	*S. pyogenes*	Interferes by releasing lipoteichoic acid from the bacterial cell surface	Sun *et al*, 1988
	Coptis chinensis	Toxins/ Adherence	*S. aureus*	Inhibitory activity against SrtA and SrtB	Oh *et al*, 2006
Epigallocatechin gallate **(39)**	*Camellia sinensis*	Toxins	*B. anthracis*	Inhibits the anthrax lethal-factor (LF) preventing MAPK-kinases cleavage and macrophages death. Delays death of mice exposed to the anthrax toxin	Dell'Aica *et al*. 2004
(-)-Catechin gallate **(40)**					
(-)-Epicatechin gallate **(41)**			*S. aureus*	Disrupts secretion of α-toxin and coagulase	Shah *et al*. 2008
β-Sitosterol-3-*O*-glucopyranoside (**42**)	*Fritillaria verticillata*	Toxins/ Adherence	*S. aureus*	Inhibitory activity against SrtA and SrtB	Park *et al*. 2005 Kang *et a*l 2006, Oh *et al*, 2006a
Psammaplin A1 (**43**)	Marine sponge *Aplysinella rhax*				
Morin (**44**)	Several plant species				
Myricetin (**45**)					
Kaempferol **(46)**					
Curcumin (**47**)	*Curcuma longa*				
Demethoxy-curcumin (**48**)	Derivate				
Bisdemethoxy-curcumin (**49**)					
Anacardic acids derivate C:11-C:16 (**50**)	Anacardiaceae	TCS	*In vitro*	Inhibits HKSPs	Kanojia *et al*, 1999
Methyl *trans*-4-hydroxycinnamate (**51**)	Derivate	TCS	*P. aeruginosa*	Inhibits T3 effector protein via GacS-GacA	Yamazaki *et al*, 2012a

T3SS: type III secretion system. TCS: two-component system

Since the discovery of the importance of T3SS for bacterial virulence in many Gram-negative bacteria, it has been an attractive target for the identification and characterization of new anti-virulence compounds (Keyser *et al*. 2008; Khokhani *et al*. 2013). In fact, several T3SS inhibitors have recently been identified, particularly from natural sources such as plants, microorganisms and invertebrates (Zetterström *et al*. 2013) (Figure 4.3 and 4.4).

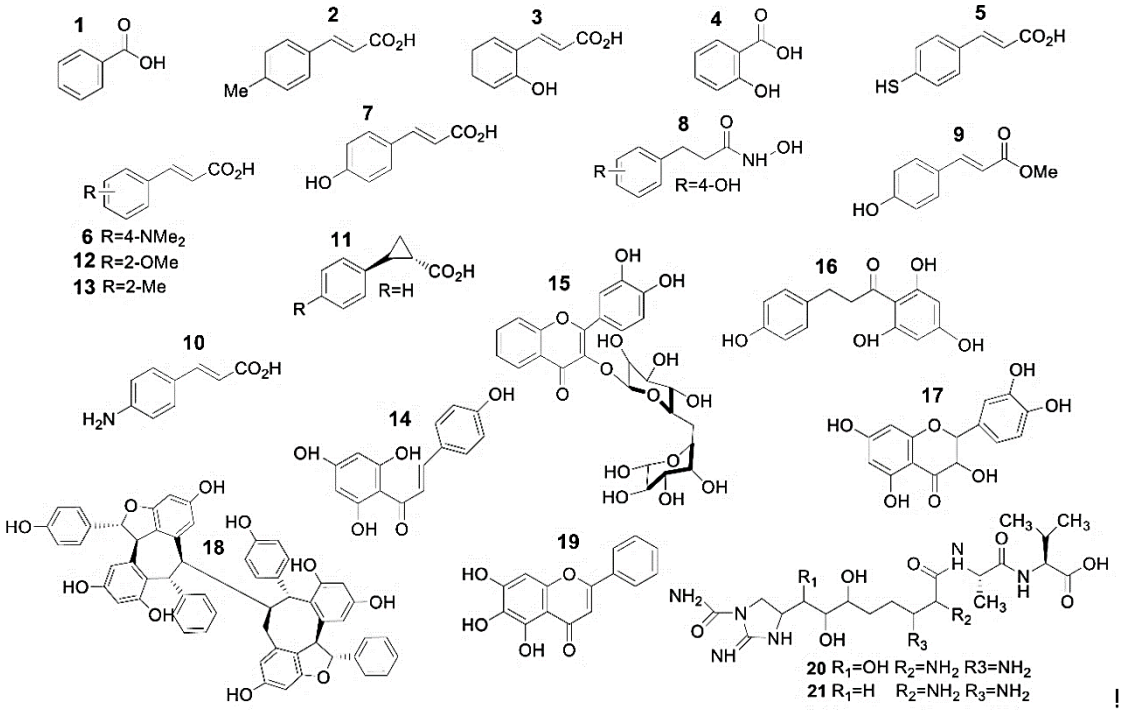

Figure 4. 3. Natural products and some derivates that inhibit bacterial secretion systems. Benzoic acid (1), 4-methoxy-cinnamic acid (2), *o*-coumaric acid (3), salicylic acid (4), *trans*-4-mercapto-cinnamic acid (5), *trans*-4-dimethylamino-cinnamic acid (6), *p*-coumaric acid (7), *trans*-4-hydroxycinnamohydroxamic acid (8), methyl *p*-coumarate (9) and *trans*-4-amino-cinnamic acid (10) *trans*-2-phenylcyclopropane-1-carboxylic acid (11), *trans*-2-methoxy-cinnamic acid (12) *trans*-2-methyl-cinnamic acid (13), chalconaringenin (14), rutin (15), phlorentin (16) quercetin (17), (-)-hopeaphenol (18) baicalein (19), guadinomine A (20) and B (21).

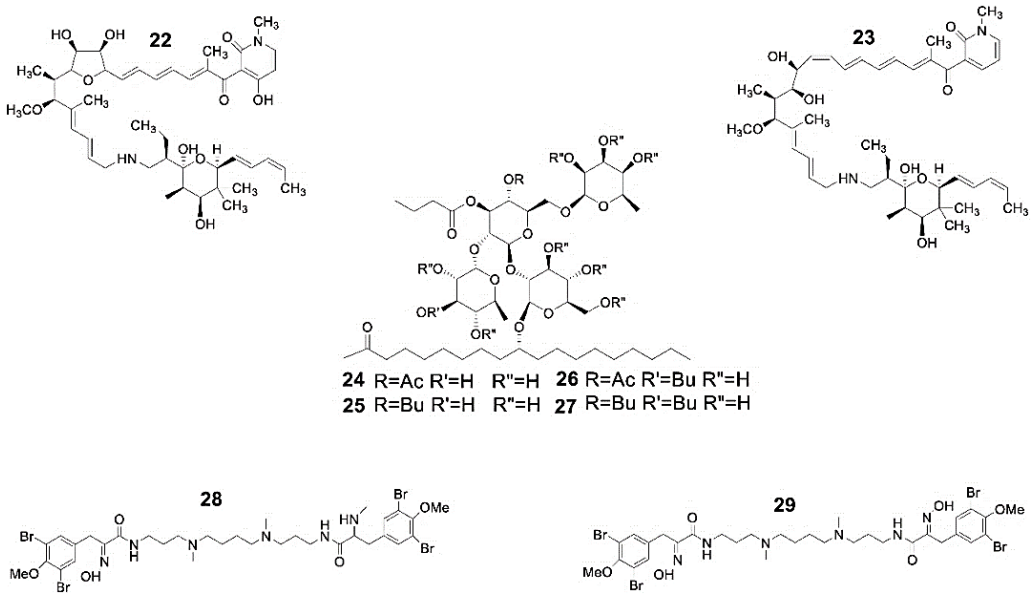

Figure 4. 4. Natural products and some derivates that inhibit bacterial secretion systems. Aurodox (22), factumycin (23), caminosides, A (24), B (25), C (26), D (27), pseudoceramide A (28) and spermatinamine (29).

Phenolic compounds are one of the secondary metabolites synthetized by plants that can play a key role in their defense against pathogens, but also a few studies indicate their potential as T3SS-blocking compounds (Zetterström *et al*. 2013). For example, in the plant pathogen *Erwinia amylovora* a causal agent of fire blight, benzoic acid (**1**) and 4-methoxy-cinnamic acid (**2**) inhibited expression of T3SS without affecting bacterial growth (Khokhani *et al*. 2013). In addition, *o*-coumaric acid (**3**), salicylic acid (**4**), *trans*-4-mercapto-cinnamic acid (**5**) and *trans*-4-dimethylamino-cinnamic acid (**6**) were found to be potent T3SS inhibitors (Khokhani *et al*. 2013).

The inhibitory effect found *in vitro* by (**2**) and (**1**) was confirmed *in vivo*, since these inhibitors reduced the hypersensitive response on tobacco leaves (Khokhani *et al*. 2013). The structure-activity study of these compounds demonstrated that the presence of electron-donating groups like in (**2**) (4-MeO group), (**5**) (4-mercapto group) and (**6**) (4-Me_2N group) is essential for exhibiting its anti-virulence properties, since if an electron-withdrawing group is present, such as

the nitro group in the 4 position, its anti-virulence effect is lost, and only some inhibition of bacterial growth is observed (Khokhani *et al*. 2013).

p-Coumaric acid (**7**) represses the expression of T3SS regulatory genes in the phytopathogenic bacteria *Dickeya dadanii*. The *para* positioning of the hydroxyl group in the phenyl ring and the double bond in the structure are important components for biological activity (Li *et al*. 2009a). The derivative *trans*-4-hydroxycinnamohydroxamic acid (**8**) was found to be a highly potent inhibitor of *D. dadantii* T3SS, more effective than (**7**) and slightly promoting bacterial growth at a high concentration (100 µM) since it can also be used as a carbon source (Li *et al*. 2015b).

Among derivatives of phenolic compounds, methyl *p*-coumarate (**9**) and *trans*-4-amino-cinnamic acid (**10**) inhibit the T3 effector protein and repress the expression of T3SS regulation genes in *P. aeruginosa* without affecting their growth (Yamazaki *et al*. 2012).

Furthermore, *o*-coumaric acid (**3**), *trans*-2-phenylcyclopropane-1-carboxylic acid (**11**), *trans*-2-methoxy-cinnamic acid (**12**) and *trans*-2-methyl-cinnamic acid (**13**) suppress the translocation of T3 proteins in *Xanthomonas oryzae* pv. *oryzae* (Xoo), possibly by T3SS-specific inhibition since other virulence factors were not suppressed (Fan *et al*. 2016). Likewise, in planta assays have shown that these compounds attenuated hypersensitive response in tobacco plants, without affecting bacterial survival, confirming their potential as T3SS inhibitors (Fan *et al*. 2016).

Among the phenolic compounds are flavonoids, a group of metabolites which exhibit diverse pharmacological activities (Pietta 2000) and can be synthesized by plants in response to microbial attack (Vargas *et al*. 2013). Tomato plants respond to *Pseudomonas syringae* pv. *tomato* infection by producing some flavonoids such as chalconaringenin (**14**), rutin (**15**), phlorentin (**16**) and quercetin (**17**), which can diminish T3SS expression (Vargas *et al*. 2013).

A tetramer of resveratrol, (-)-hopeaphenol (**18**), obtained from extracts of *Anisoptera thurifera* and *A. polyandra* leaves, inhibits the secretion and expression of T3SS effector proteins in *Yersinia pseudotuberculosis* and *P. aeruginosa*, and also affects their translocation into HeLa cells in a dose-dependent manner without affecting growth (Zetterström *et al*. 2013).

Flavonoids present in traditional Chinese medicines such as baicalein (**19**) from *Scutellaria baicalensis* and quercetin (**17**) showed T3SS inhibitory activity against *Salmonella enterica* serovar Typhimurium (Tsou *et al*. 2016). This bacterium, has two T3SSs encoded by pathogenicity island SPI-1 and SPI-2 (Collazo & Galán 1997; Hansen-Wester & Hensel 2001). SPI-1 is required for initiating bacterial uptake by host cells, while SPI-2 ensures that the bacteria can grow inside intracellular compartments (Keyser et al. 2008). In contrast, compounds (**19**) and (**17**) inhibit SP1-1 T3SS by blocking the synthesis and assembly of the needle complex, rather than by inhibiting protein secretion. Hence, the specific target of these compounds might be type III secreted proteins or protein translocases (Tsou *et al*. 2016).

With derivatives of (**19**), it was demonstrated that the 1,2-catechol moiety, flavone scaffold, and phenolic hydroxyl groups are important for its inhibitory activity, since methylated or truncated forms were inactive (Tsou *et al*. 2016). Also, the reactive 1,2 catechol motifs in both compounds covalently modify SPI-1 T3SS substrates, possibly resulting in their functional inactivation (Tsou *et al*. 2016).

The guadinomides are six new compounds isolated from the actinomycete *Streptomyces* sp. K01-0509 and were evaluated as T3SS inhibitors against enteropathogenic *E. coli*. The results obtained indicated that guadinomines A (**20**) and B (**21**) showed the strongest inhibitory activity and do not affect bacterial growth, indicating that the amino groups are important for the activity.

In comparison, while guadinomic acid has no activity, the cyclic guanidine moiety alone is not sufficient for achieving inhibitory effect (Iwatsuki *et al*. 2008).

Similarly, in other strains of *Streptomyces* sp. K06-0806 and K07-0034 two linear polyketide compounds were isolated, aurodox (**22**) and their analog factumycin (**23**), which exhibited inhibitory activity on T3SS secretion proteins in *Citrobacter rodentium* without affecting bacterial growth. Compound (**22**) was the most active, suggesting that the cyclized partial structure and/or the hydroxy group at C-2 of the pyranose ring is critical to achieving inhibition of the T3SS function. Administration of this T3SS inhibitor increased the survival of mice that had received a lethal dose of *C. rodentium,* which causes colonic hyperplasia in a T3SS-dependent manner. Notably, this is the first report of successful inhibition *in vivo* that may provide the starting material for novel T3SS inhibitors (Kimura *et al*. 2011).

Complex glycolipids such as caminoside A (**24**), B (**25**), C (**26**) and D (**27**) isolated from extracts of the marine sponge *Caminus sphaeroconia* also have inhibitory activity against T3SS expression in *E. coli* at a concentration of 20 μM without killing the bacteria (Linington et al. 2006a; Linington *et al*. 2006b).

Caminosides possess several structural features not found in other sponge glycolipids to date, such as a fully substituted glucose residue, as well as a 6-deoxytalose residue, rarely seen in nature. Aglycon also contains several unusual features, including the C_{19} linear chain and the methyl ketone terminus, which may contribute to their inhibitory activity (Linington *et al*. 2006a).

The marine sponge *Pseudoceratina* sp. was the source of new bromotyrosine alkaloids called pseudoceramides *A-D,* along with a known natural product, spermatinamine (**29**). These compounds were screened to test their inhibitory activity on the T3SS of *Yersinia pseudotuberculosis*. Remarkably, pseudoceramide B (**28**) and **29** inhibited T3 proteins secretion.

The data also revealed that the intact bromotyrosyl-spermine-bromotyrosyl sequence and methoxy groups may be essential for the inhibition (Yin *et al*. 2011).

Several natural products capable of inhibiting production of fimbriae in some genera of bacteria have been identified (Figure 4.5). Specifically, phloretin (**16**), ginkgolic acids C15:1 (**30**), C17:1 (**31**), coumarin (**32**), umbelliferone (**33**), cinnamaldehyde (**34**) and eugenol (**35**) reduce their production in enterohemorrhagic *E. coli* O157:H7 (Kim *et al*. 2016; Lee *et al*. 2011b; Lee *et al*. 2014b).

Transcriptomic analysis by DNA microarrays and qRT-PCR demonstrated that these compounds repress the expression of *csgA* and *csgB* genes involved in the synthesis of curli, the major protein component of the extracellular matrix that allows the formation of three-dimensional structures such as biofilms (Kim *et al*. 2016; Lee *et al*. 2011; Lee *et al*. 2014).

Also, asiatic acid (**36**) and ursolic acid (**37**) reduce the formation of fimbriae in different strains of uropathogenic *E. coli* (Wojnicz *et al*. 2013). However, an increase in the number of hydroxyls in their structures, specifically in ring A (**36**), promotes a bactericidal effect, perhaps by promoting changes in hydrophobicity that facilitate its free diffusion into the bacterial cell (Wojnicz *et al*. 2013).

Some alkaloids also disrupt adhesion fimbriae and other virulence factors by non-QQ-mediated mechanisms. In *Streptococcus pyogenes,* sublethal concentrations of the alkaloid berberine (**38**) interfere with its adherence by releasing lipoteichoic acid from the bacterial cell surface, as well as by dissolving lipoteichoic acid-fibronectin complexes (Sun *et al*. 1988a). Also, this compound decreased adhesion of uropathogenic *E. coli* to erythrocytes and epithelial cells, and it is suggested that this effect may be mediated by the selective suppression of synthesis and assembly of fimbriae (Sun *et al*. 1988b).

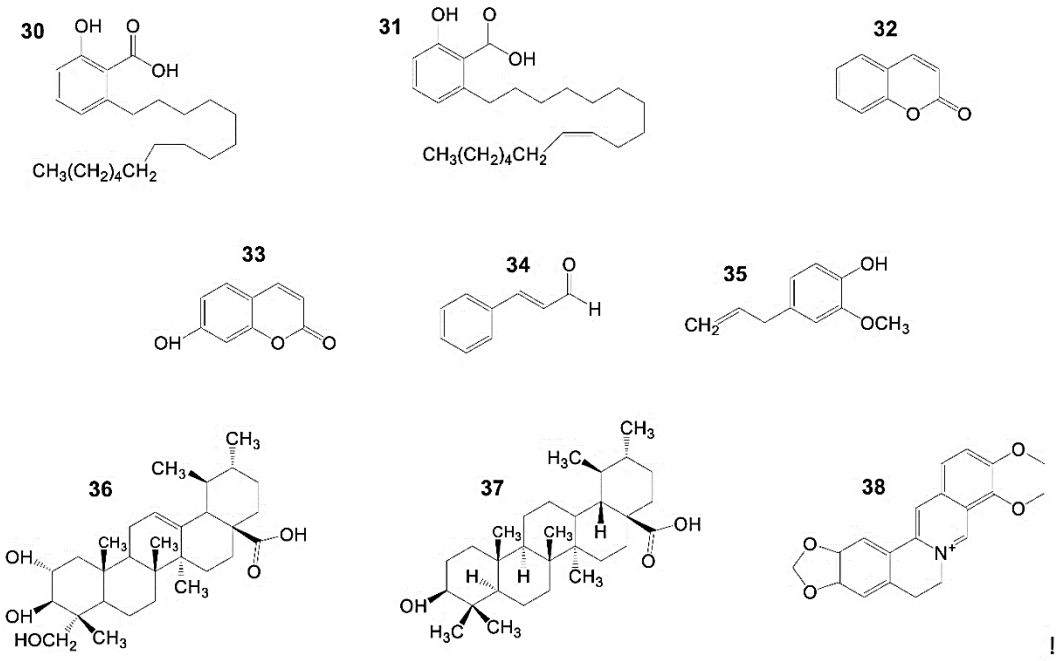

Figure 4.5. Natural products that inhibit formation of fimbriae. Ginkgolic acids C15:1 (30) and C17:1 (31), coumarin (32), umbelliferone (33), cinnamaldehyde (34), eugenol (35), asiatic acid (36), ursolic acid (37) and berberine (38).

4.6. Natural products as inhibitors of toxins, key enzymes and two component systems.

Toxins are one the main virulence factors used by bacteria that cause disease in the host. Epigallocatechin gallate (**39**) and catechin gallate (**40**) directly inhibit the anthrax lethal-factor (LF) produced by *Bacillus anthracis* which has a key role in the development of anthrax (Dell'Aica *et al*. 2004) (Figure 4.6). LF is a zinc metalloprotease that directly affects MAPK-signalling kinases that are essential for transmitting signals in eukaryotes.

The compounds (**39**) and (**40**) block the activity of LF, preventing MAPK-kinase cleavage and macrophage death (Dell'Aica *et al*. 2004). In the case of (**39**), it also delays death of mice exposed to the anthrax toxin (Dell'Aica *et al*. 2004). It is noteworthy that, although other catechins were evaluated, the presence of a galloyl group in the structure seems to be essential for this anti-virulence activity.

Also, (-)-epicatechin gallate (**41**) disrupted the secretion of virulence-related proteins by *S. aureus* such *α*-toxin and coagulase (Shah *et al*. 2008). This is likely due to changes in the physical nature of the bacterial membrane, as well to the intercalation of galloyl catechins in the cytoplasmic membrane, possibly interfering with membrane-associated signal transduction processes (Shah *et al*. 2008).

The sortase enzymes are transpeptidases responsible for anchoring surface protein and assembly of pili to the peptidoglycan cell wall layer of Gram-positive bacteria (Maresso & Schneewind 2008). Four different classes of sortase exist, namely SrtA-D (Maresso & Schneewind 2008). SrtA and SrtB are responsible for the cell wall anchoring proteins involved in bacterial adhesion, while SrtC, assemble pili on the bacterial surface (Maresso & Schneewind 2008).

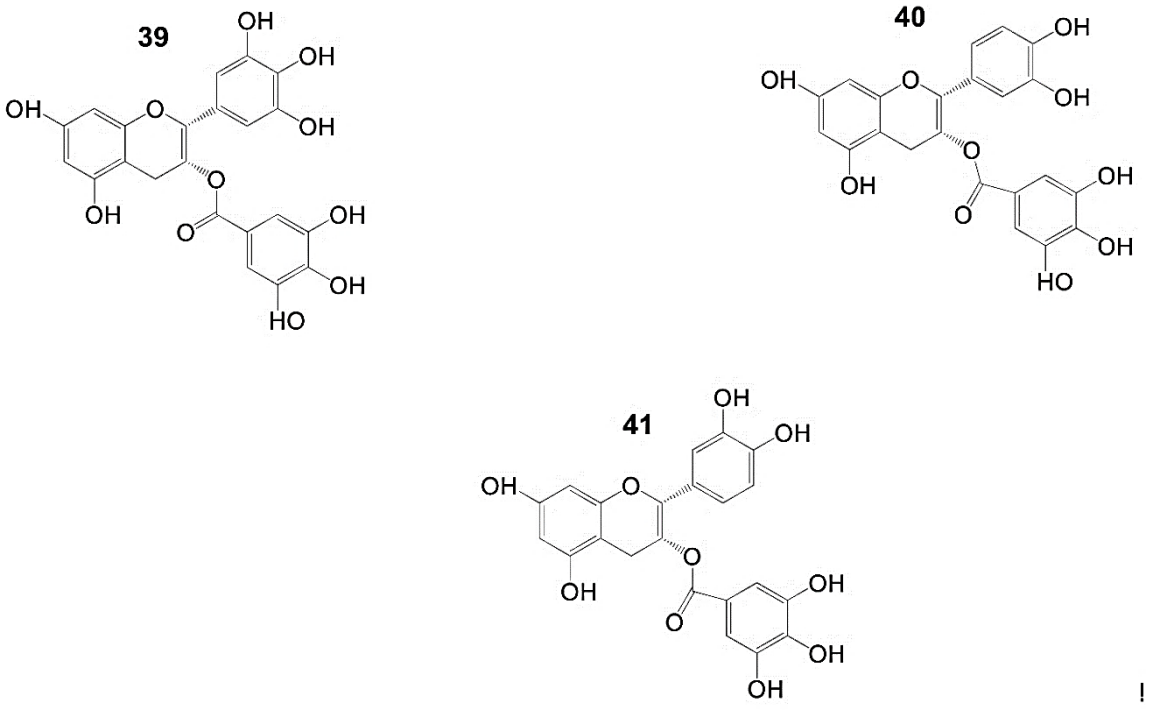

Figure 4. 6. Natural products that inhibit bacterial toxins. Epigallocatechin gallate (39), catechin gallate (40) and (-)-epicatechin gallate (41).

β-sitosterol-3-*O*-glucopyranoside (**42**), berberine (**38**), psammaplin A1 (**43**) (Oh et al., 2006b)**,** morin (**44**), myricetin (**45**), quercetin (**17**), kaempferol **(46)** (Kang *et al*. 2006), curcumin (**47**), dimethoxy-curcumin (**48**) and bisdemethoxy-curcumin (**49**) (Park et al., 2005) exhibited potent inhibitory activity against *S. aureus* cell adhesion to fibronectin (a glycoprotein present in vertebrates) and showed potent inhibitory activity against SrtA and SrtB without exhibiting antibacterial activities at the highest concentration tested (minimum inhibitory concentration >300 µM) (Figure 4.7) (Park *et al*. 2005; Kang *et al*. 2006).

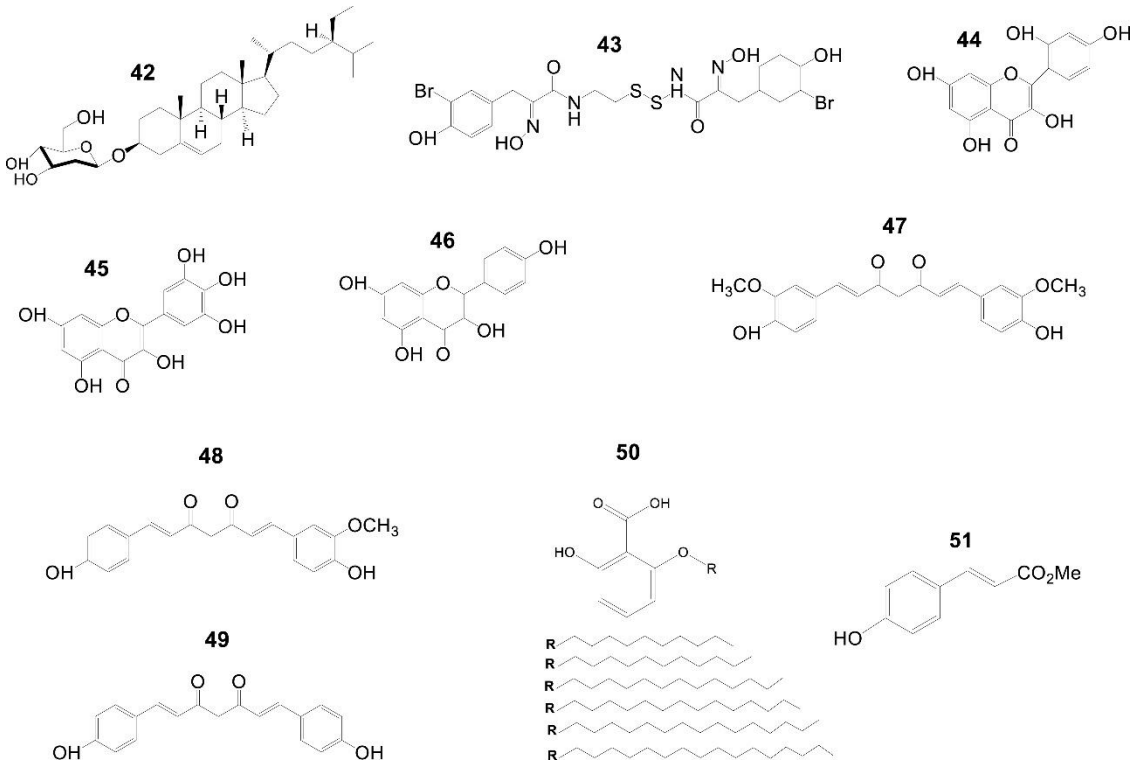

Figure 4. 7. Natural products and some derivates that inhibit the sortase enzyme or two-component system. β-Sitosterol-3-*O*-glucopyranoside (42), psammaplin A1 (43), morin (44), myricetin (45), kaempferol (46), curcumin (47), dimethoxy-curcumin (48), bisdemethoxy-curcumin (49)**,** 6-oxa isosteres anacardic acids C:11 to C:16 (50) and methyl *trans*-4-hydroxycinnamate (51).

In the case of (**43**), it was found that the side chain moiety glucopyranoside is important because, if removed from the molecule, its activity is significantly reduced (Oh *et al*. 2006). Similarly, for the case of flavanols, the co-occurrence of a hydroxyl group at C-3 of the C ring and meta-hydroxy groups at C-2´ and C-4´ of the B ring are required for the activity (Kang *et al*. 2006; Maresso & Schneewind 2008).

Pathogenic bacteria can respond and adapt to a variety of hostile environments in order to colonize their host; in this process, the two-component systems (TCS) have a very important role(Mitrophanov & Groisman 2008). TCSs are response regulators formed by a protein localized in the cytoplasmic membrane called histidine-kinase-sensory protein (HKSP), which acts as an environmental sensor that is ATP-dependently activated (Mitrophanov & Groisman 2008). HKSP then activates a response regulator protein (RR) found in the cytoplasm which is responsible for recognizing DNA sequences that modulate the expression of genes involved in several functions such as virulence and biofilm formation in pathogenic bacteria (Mitrophanov & Groisman 2008; Worthington *et al*. 2013).

Although some TCSs are part of the QS systems (sensing signals), this class of regulators is broader and is also known to regulate others phenomena, including resistance to antibiotics. They are activated by environmental factors such as nutrient levels, pH, redox state, osmotic pressure, etc. (Worthington *et al*. 2013). An important feature is that TCSs have not been detected in mammal cells and so are a suitable specific target for the treatment of bacterial infections (Stephenson & Hoch 2002; Worthington *et al*. 2013). Many bacterial species encode, within their genomes, a large number of TCSs, which have roles in diverse behaviors (Worthington *et al*. 2013).

In this regard, some natural products that inhibit these systems have been described (Figure 4.7). For example, the anacardic acid derivate (6-oxa isosteres) C:11-C:16 (**50**) inhibited HKSPs (KinA/SpoOF and NRII/NRI) (Kanojia *et al*. 1999).

Interestingly, AA with alkyl chains outside this range were not active (Kanojia *et al*. 1999). Likewise, for this activity the presence of the carboxyl group is important, as C:12 and C:14 completely lose their effect, and the presence of phenolic OH partially restores it (Kanojia *et al.* 1999). Other phenolic compounds such as the coumaric acid derivates (*trans*-4-aminocinnamic acid (**10**) and methyl *trans*-4-hydroxycinnamate (**51**) also inhibited the T3 effector protein of *P. aeruginosa* via a GacS-GacA TCS (Yamazaki *et al*. 2012).

4.7. Concluding remarks

In the past decade, several natural products with anti-virulence properties have been identified. These compounds have diverse targets at different regulation levels. However, although QS is considered the primary regulator of bacterial virulence in several pathogenic bacteria, recent evidence indicates that, during the infection process within the host, the overall regulation of virulence is more complex.

Hence, we can divide natural products with anti-virulence properties into two groups: those that act by inhibiting the QS (QQ) and those that act on QS independent regulatory pathways or that directly inhibit individual virulence factors. We named these factors here "DIIVF" (*direct inhibitors of individual virulence factors*).

The downregulation of some virulence factors by QS and the effect on QS, exerted by yet unknown external factors (present during the processes of infection in the host), lead us to suspect that the use of individual natural products with QQ activity will not be sufficient for fully blocking

overall virulence in bacteria and that a combination of several compounds with diverse targets is maybe needed to increase their effectiveness (Anand & Rai 2013).

Although recently it was demonstrated that a better antimicrobial effect can be achieved by combining QQ molecules with commercial antibiotics (Brackman *et al*. 2011; Christensen *et al*. 2012; Furiga *et al*. 2015; Dasko 2016), whether the combination of different molecules with anti-virulence properties can provide better results for treating infections, has not yet been explored.

Hence, we propose a possible benefit of using therapies that act simultaneously on these "two groups" (QQ and DIIVF) and we suggest that it is feasible to increase the efficiency in global virulence quenching by use of these combinations. Moreover, it is attractive to identify more natural products that have more than one anti-virulence target in the same bacterial system. This would have the advantage of inducing fewer side effects than those caused by the simultaneous administration of various drugs.

4.8. Acknowledgements

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CONCLUSIONES GENERALES

Se identificó actividad antivirulencia *in vitro* de fitoquímicos presentes en la corteza de dos especies vegetales utilizadas en la Medicina Tradicional Mexicana y moléculas sintéticas halogenadas, sintetizadas por el grupo de trabajo, mediante la I-SPQ.

El potencial I-SPQ de extractos vegetales es compleja, ya que en este trabajo se muestra que la actividad del extracto puede variar al fraccionarse, inhibiendo o estimulando la virulencia bacteriana, lo cual puede estar estrechamente relacionado con la naturaleza química de los compuestos presentes y la especie bacteriana evaluada.

En el caso de las moléculas sintéticas halogenadas, la inhibición de los factores de virulencia evaluados fue variable, sin embargo, afectan notablemente la formación de biopelículas y motilidad bacteriana mediante swarming en cepas de referencia y resistentes a antibióticos de *P. aeruginosa*.

Los resultados obtenidos *in vitro* e *in vivo*, sugieren que para evaluar de una manera más clara la I-SPQ de las moléculas de interés es importante utilizar un amplio rango de concentraciones, ya que dependiendo de la dosis aplicada pueden actuar como activadoras o inhibidoras de los SPQ, además de ser potencialmente bactericidas.

La mayoría de los compuestos evaluados naturales y/o sintéticos inhibieron factores de virulencia regulados por PQ en diferentes sistemas bacterianos, lo que indica su potencial como moléculas antivirulencia. Estudios posteriores se centrarán en evaluar su efectividad un modelo de infección *in vivo*, así como su uso en combinación con otros inhibidores de virulencia no regulados por quorum, como es el caso del sistema de secreción tipo 3 (SST3), para ampliar su uso y eficacia en el tratamiento de diferentes infecciones bacterianas.