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

NAYBI MUÑOZ-CÁZARES¹, SILVIA AGUILAR-RODRÍGUEZ²; RODOLFO GARCÍA-CONTRERAS³, MARCOS SOTO-HERNÁNDEZ¹, MARIANO MARTÍNEZ-VÁZQUEZ⁴, MARIANA PALMA-TENANGO¹, FRANCISCO JAVIER PRADO-GALBARRO⁶, AND ISRAEL CASTILLO-JUÁREZ^{5*}

Botanical Sciences
96 (3): 415-425, 2018

DOI: 10.17129/botsci.1902

Received:
November 1st, 2017
Accepted:
April 26th, 2018
Associated editor:
Salvador Arias

¹ Posgrado en Botánica, Colegio de Postgraduados, Texcoco, Estado de México, México.

² Laboratorio de Botánica, UMF, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlanepantla, Estado de México, México.

³ Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad de México, México.

⁴ Instituto de Química, Universidad Nacional Autónoma de México, Circuito exterior, Ciudad Universitaria, Ciudad de México, México.

This is an open access article distributed under the terms of the Creative Commons Attribution License CC BY-NC (4.0) international.

Abstract

Background: Inhibition of quorum sensing systems (QSS-I) is a novel strategy in the treatment of bacterial infections. To date, plants are the major source of metabolites with this inhibitory activity. Thus, species of Mexican flora can be important resources for obtaining metabolites with QSS-I activity.

Hypothesis: We hypothesized that extracts from species of the genus *Ceiba* have metabolites with inhibitory activity against bacterial quorum sensing systems.

Species studied: *Ceiba pentandra* (L.) Gaertn. and *Ceiba aesculifolia* (Kunth) Britten & Baker f. (Malvaceae).

Study site and years of study: We collected *Ceiba* bark in the municipalities of Tierra Blanca, Veracruz, and Acatlan, Oaxaca, in August 2013.

Methods: We determined the effect of extracts from *C. aesculifolia* and *C. pentandra* against QSS-regulated phenotypes of *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. Extracts were fractionated and the main metabolites were identified. As support in the identification of the species, we carried out an anatomical study of the bark.

Results: Hexane and dichloromethane extracts of both species of *Ceiba* exhibited QSS-I activity. We identified four fractions rich in terpene and sterol compounds with the ability to attenuate virulence factors in *P. aeruginosa*. The histological analysis appears to support the presence of some differences in the barks that can facilitate identification of the two species.

Conclusions: The extracts and fractions of the two species of *Ceiba* are sources of phytochemicals with the ability to regulate bacterial quorum sensing systems positively or negatively.

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

Resumen

Antecedentes: la inhibición de los sistemas de percepción de quórum (I-SPQ) es una nueva estrategia en el tratamiento de infecciones bacterianas. Hasta la fecha, las plantas son la principal fuente de metabolitos con esta actividad inhibidora. Por lo tanto, las especies de flora mexicana pueden ser fuentes importantes para obtener metabolitos con actividad I-SPQ.

Hipótesis: los extractos de especies del género *Ceiba* tienen metabolitos con actividad inhibidora contra los sistemas de percepción de quórum bacteriano.

Especies estudiadas: *Ceiba pentandra* (L.) Gaertn. y *Ceiba aesculifolia* (Kunth) Britten & Baker f. (Malvaceas).

Lugar de estudio y años de estudio: Recolectamos las cortezas de *Ceiba* en los municipios de Tierra Blanca, Veracruz y Acatlán, Oaxaca, en agosto de 2013.

Métodos: Determinamos el efecto de extractos de *C. aesculifolia* y *C. pentandra* contra fenotipos regulados por SPQ de *Chromobacterium violaceum* y *Pseudomonas aeruginosa*. Los extractos se fraccionaron y se identificaron los principales metabolitos. Como apoyo en la identificación de la especie, realizamos un estudio anatómico de la corteza.

Resultados: los extractos de hexano y diclorometano de ambas especies de *Ceiba* exhibieron actividad I-SPQ. Identificamos cuatro fracciones ricas en compuestos de la clase de los terpenos y esteroides con la

capacidad de atenuar los factores de virulencia en *P. aeruginosa*. El análisis histológico parece apoyar la presencia de algunas diferencias en las cortezas que pueden facilitar la identificación de las dos especies.

Conclusiones: Los extractos y fracciones de las dos especies de *Ceiba* son fuentes de fitoquímicos con la capacidad de regular positiva o negativamente los sistemas de percepción de quórum bacteriano.

Palabras clave: resistencia a los antibióticos, comunicación bacteriana, plantas mexicanas, pochote, sistemas de percepción de quórum.

An emerging problem associated with the indiscriminate use of antibiotics is selection pressure, which results in bacteria with high levels of resistance (Fischbach & Walsh 2009). Therapeutic options are becoming limited, a serious problem that urgently needs to be addressed (Roy *et al.* 2011). Among new anti-virulence strategies to combat resistant bacteria, inhibition of bacterial quorum sensing systems is the most frequently proposed and studied (Muñoz-Cazares *et al.* 2017).

Bacterial communication, or quorum sensing (QS), is a regulatory mechanism that depends on population density and promotes multicellular behaviour of bacteria. It is carried out by quorum sensing systems (QSS), which consist in the production, diffusion, detection and responses to chemical signaling molecules known as autoinducers that play a fundamental role in the expression of some phenotypes in terms of their 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), which is the new target for antimicrobial chemotherapy (Zhang & Dong 2004, Adonizio *et al.* 2006). Unlike antibiotics, quorum sensing system inhibition (QSS-I) represses the expression of virulence factors and biofilms without affecting 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 can eliminate the infection (Roy *et al.* 2011).

New investigations have focused on discovering agents derived from synthetic and natural products to handle bacterial pathogenesis by means of QSS-I (Pan & Ren 2009). In Mexico around 4,000-5,000 species of plants have medicinal properties which are frequently used to treat disorders (Espinosa *et al.* 2008, Valdivia-Correa *et al.* 2016). The bark of “pochote” or “pochotl” trees is widely used in therapeutic applications. The name “pochote” is used in the traditional nomenclature to refer several species of the genus *Ceiba* distributed in different regions of Mexico (Canales *et al.* 2005, Pennington & Sarukhán 2005, Avendaño *et al.* 2006). *Ceiba pentandra* (L.) Gaertn. and *Ceiba aesculifolia* (Kunth) Britten & Baker f. (Malvaceae) are the best-known in the national territory.

Although *C. pentandra* is native to Central America, it has been introduced to various regions of the world (Gibbs & Semir 2003, SEMARNAT 2013) and extracts from its seeds, leaves, bark and fruits have been reported to have antibacterial activity (Anosike *et al.* 2012, Osuntokun & Adeoye 2017, Parulekar 2017). Also, compounds such as naphthaquinones, sesquiterpenoids, isoflavones, steroids, fatty acids and different glucosides in the extracts have been isolated (Noreen *et al.* 1998, Ngounou *et al.* 2000, Ueda *et al.* 2002, Kishore *et al.* 2003, Refaat *et al.* 2013), but only sesquiterpene lactones from the root bark showed bactericidal activity (Rao *et al.* 1993).

Ceiba aesculifolia is native to the Mexican tropical dry forest (Niembro *et al.* 2010) in central Mexico. Its bark is used to cure skin infections and wounds (Canales *et al.* 2005, Orozco *et al.* 2013, Franco *et al.* 2016). Methanolic extracts from the bark and fibers of mature fruits showed bactericidal activity. In these extracts phenolic compounds such as coumarins, flavonoids and phenylpropanoids were found to be the major components, together with isoflavones, sterols, terpenes and fatty acids (Orozco *et al.* 2013, Franco *et al.* 2016).

Commercial distribution of the *Ceiba* bark has not been documented. The bark can be found in traditional medicine stands in native markets, sold in pieces as “pochote”, with no differentiation between species. It is, however, important to differentiate species and their medicinal properties. Thus, methods of identification using anatomical characteristics to distinguish species of medicinal importance are needed to support pharmacognosy studies (Rivera-Arce *et al.* 2007, Rosas-Acevedo *et al.* 2011). In this study, we evaluated the effectiveness of *C. aesculifolia* and

⁵ Investigador Cátedras-CONACYT, Posgrado en Botánica, Colegio de Postgraduados.
⁶ Agencia de Evaluación de Tecnologías Sanitarias, Instituto de Salud Carlos III, Madrid, España.
 *Corresponding author: israel.castillo@colpos.mx
 Author contributions
 Naybi Muñoz-Cázares performed the experiments, analyzed the data and wrote the paper.
 Silvia Aguilar-Rodríguez contributed with the histological methods for the identification of *Ceiba* barks.
 Rodolfo García-Contreras performed the experiments with *Pseudomonas aeruginosa*, analyzed the data and reviewed drafts of the paper.
 Mariano Martínez-Vázquez contributed with the phytochemical analysis of *Ceiba* barks.
 Marcos Soto-Hernandez contributed with the phytochemical analysis of *Ceiba* barks and reviewed drafts of the paper.
 Mariana Palma-Tenango edited the images and reviewed drafts of the paper.
 Francisco Javier Prado-Galbarro supported the statistical analysis of the paper.
 Israel Castillo-Juarez conceived and designed the experiments, analyzed the data and wrote the paper.

C. pentandra barks to inhibit QSS in *Chromobacterium violaceum* and *Pseudomonas aeruginosa* to propose them as possible source of anti-virulence metabolites. In addition, an anatomical study was made to support identification of the species.

Materials and methods

Plant material. Stem bark of *Ceiba aesculifolia* was collected in the municipality of Tierra Blanca, state of Veracruz, Mexico (18° 34.189' N and 96°22.690'W). For *C. pentandra*, samples were obtained in the municipality of Acatlán, state of Oaxaca, Mexico (18° 32.677' N and 96° 36.336' W). Botanical identification was carried out by Dr. Antonio Guízar Nolasco (DICIFO/UACH), and two voucher specimens were deposited at the Herbarium of the Universidad Autónoma Chapingo (CHAP), registration numbers *C. pentandra* 66,486 and *C. aesculifolia* 66, 487.

Extract preparation and fractionation. The air-dried and powdered bark (1.0 kg) of the two *Ceiba* species was sequentially extracted with hexane (Hex), dichloromethane (D), and methanol (MeOH) (J.T. Baker®). The solvent was evaporated under low pressure, yielding CpHex 3.37 g, CpD 5.22 g, CaHex 2.42 g and CaD 4.32g of crude extracts. The hexane and dichloromethane extracts were subjected to a vacuum column chromatography using silica 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 CaHex, 12 fractions from CaD, CpHex six fractions and 12 final fractions in CpD. The obtained fractions were concentrated and analyzed by thin layer chromatography (TLC). TLC analyses were performed according to conventional techniques, using 0.25 mm thick aluminum plates (60 F254 Merck®). The plates were visualized under UV light (254 nm) and subsequently developed with 2 % vanillin-10 % H₂SO₄ in ethanol.

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

Anti-quorum sensing activity in *C. violaceum*. Two biomonitor strains were used. ATCC553 is a wild type strain that synthesizes violacein, a QS purple pigment, whose production is regulated by the C4 and C6 homoserine lactones autoinducer molecules (AHL). The other strain, CV026, is a mini Tn5 mutant-indicator derived from the wild type CV31532 strain; it is unable to synthesize its own AHL but retains the ability to respond against exogenous AHL.

The effect of extracts and fractions on the QS controlled production of violacein was determined using the wild-type ATCC553 strain, while the potential toxic effects on growth was monitored using the non-pigmented CV026 strain. To determine whether violacein was inhibited and bacterial growth was affected, a multi-well system assay was conducted. Cultures were adjusted to an optical density of 600 nm = 0.05 (10⁵ CFU/mL⁻¹) (Multiskan Spectrum) and seeded (200 µL) in each well of 96-well microtiter polystyrene plates (Corning®). 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.

Plates were incubated at 25 °C with constant shaking at 120 rpm for 48 h. DMSO and LB medium were used as negative controls and anacardic acid mixture (AA) 100 µg/mL as positive control (Castillo-Juárez *et al.* 2013). The violacein obtained after drying the culture medium was resuspended in 200 µL of DMSO and the absorbance was measured at 590 nm. To calculate the percentage of inhibition, absorbance of the negative controls was considered 100 % violacein production. Bacterial growth was determined by absorbance of the cultures at 600 nm. Inhibition percentage was calculated by subtracting the absorbance of the extracts and fractions from that of the cultures. The value obtained in LB medium controls was considered 100 % growth.

Anti-quorum sensing activity in *Pseudomonas aeruginosa*. To test the expression of virulence factors, a PA14 wild type was used. For all experiments, precultures were initiated aerobically

from single colonies in LB broth at 37 °C, shaking at 200 rpm for 20 h. To evaluate QSS-I, overnight cultures were again inoculated in LB broth at initial turbidity of $OD_{600} \sim 0.05$ (UV-1800, Shimadzu). Extracts and fractions were then dissolved in DMSO and 5-10 μ L added to 5 mL of the cultures with final concentrations of 128 to 500 μ g/mL and incubated for 7 h. Bacterial growth was measured every two hours at 600 nm. DMSO was used as negative control, and the production of all the virulence factors was normalized by growth (absorbance 600 nm). After incubation time, cells were centrifuged and the supernatant was used to determine expression of QS-controlled virulence factors. For extracts, at least three independent cultures were included, while for fractions one assay was done with two replicates.

Pyocyanin production was determined spectrophotometrically after extraction from the cultures with chloroform and a further extraction with hydrochloric acid 0.2 N. The pyocyanin concentration was estimated from the peak to an optical density at 520 nm with a millimolar extinction coefficient of $2.46 \text{ mM}^{-1} \text{ cm}^{-1}$ (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).

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

Histological methods. Segments of the inner and outer bark ($3 \times 2 \text{ cm}$) of four *C. aesculifolia* and *C. pentandra* individuals were obtained at a height of 1.30 m from the main stem. Subsequently, the segments were softened in a solution of ethyl alcohol, glycerin and water (GAA, 1:2:3) for a period of 30 days. For the microtechnical procedure transverse, tangential and radial sections (20-30 μ m) were made, using a sliding microtome. In the case of tangential sections, serial cuts were made from the bark to the vascular cambium. Sections were stained with safranin-fast green to be mounted in synthetic resin (Ruzin 1999). *Ceiba* bark was described anatomically following the recommendations of Trockenbrodt (1990) and Angyalossy *et al.* (2016). Images were obtained using the analyzer elements NIS-BR 2.33 (Nikon corporation 1991-2006). The general images were prepared using a Lucida camera at 1X on a Nikon Labophot-2 microscope.

Statistical analyses. The results are presented as the average and standard deviation of at least three independent experiments. For extracts and fractions against violacein production in *C. violaceum* the Student's *t* test was used, while fractions against *P. aeruginosa* virulence factors were analyzed by One-way with Bonferroni test. These analyses were done in IBM-SPSS 22v software.

Results

Inhibitory activity of the extracts in production of QSS-regulated C. violaceum and P. aeruginosa phenotypes. QSS-I was observed mainly in the extracts CaD, CpHex and CpD, which inhibited violacein production 60 %, while non-significative effects on CV026 strain growth were found (Figure 1). CaMeOH, CpMeOH and CaHex extracts reduced pigment production but showed significant effect on CV026 strain growth (Figure 1). In these assays, a AA mixture was employed as a positive control since a previous study reported that this compound inhibits the production of violacein (Castillo-Juárez *et al.* 2013). Only extracts from *C. aesculifolia* at the highest dose (384 μ g/mL) significantly inhibited *P. aeruginosa* virulence factors. CaHex decreased elastolytic activity (26.5 %) and CaD inhibited pyocyanin, pyoverdine and elastolytic activity up to 30 %, without affecting bacterial growth (data not shown).

Effect of the fractions on production of QSS-regulated C. violaceum and P. aeruginosa phenotypes. Only fractions with a yield sufficient for conducting the assays were selected. The fractions with significant QSS-I, as well as the yield and system of elution, are shown in Table 1. The fractions exhibited different activities stimulating or inhibiting virulence; ICaHex,

Figure 1. Inhibition of violacein production by *C. aesculifolia* and *C. pentandra* bark extracts. Black bars represent bacterial growth (mutant CV026) and white bars represent violacein production (CV12472 WT). AA = anacardic acid mixture. Hex = hexane, D = dichloromethane, MeOH = methanol. * $P < 0.05$ by Student's *t*-test compared with control.

VICpHex, ICpHEX and IIICpD were the most active fractions against the pyocyanin and alkaline protease activity of *P. aeruginosa*. Interestingly, whereas these fractions inhibit virulence factors in this bacterium, *C. violaceum* stimulates production of violacein or shows a discrete inhibitory effect.

Dose-response QSS-I effect of fractions on the alkaline protease activity of P. aeruginosa. The dose-response effect of the active fractions ICaHex, VICaD, ICpHex and IIICpD on alkaline protease activity and growth of *P. aeruginosa* was analyzed, and the effect was observed only for ICpHex and ICaBHex (Figure 2). It should be noted that the fractions at higher doses of 250 μg/mL presented problems of solubility in the cultures, a phenomenon that may be responsible for the unclear dose-response effect in some fractions.

Principal groups of metabolites present in the active fractions. The active fractions were screened for the presence of common classes of plant secondary metabolites. The four fractions analyzed are composed mainly of terpene-type metabolites (Table 2). Triterpene and steroidal type compounds were detected in ICaHex, VICaD and ICpHex, whereas flavonoids were found in VICaD and ICpHex.

Anatomical differences between C. aesculifolia and C. pentandra barks. Clear differences between the *Ceiba* barks (Figure 3) were seen in cross-section. In *C. aesculifolia*, the prickles are stratified (stratified phellem): 2-3 layers of cells with clear lumens and thin walls alternate with numerous layers of thicker-walled cells (Figure 3B). This stratification is repeated (Figure 3A) up to more than five times in some prickles. In *C. pentandra*, the prickles are smaller and form

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

Specie	Extract	Fraction/ Solvent	Yield (mg)	Inhibition of QS (%)							
				<i>C. violaceum</i> (200 µg/mL)			<i>P. aeruginosa</i> (500 µg/mL)				
				VN	<i>sd</i>	GW	PY	<i>sd</i>	AP	<i>sd</i>	
<i>Ceiba aesculifolia</i>	H	I 9H:1EA	485	+3	26.1	+3	57.4**	1.7	79.5**	7.7	
		II 8H:2EA	352	42*	7.9	-7	+31.9	8.7	+14.2**	27.2	
		III 6H:4EA	282	45*	7.6	-3	18.8	7.8	38.6	18.9	
		IV 3H:7EA	129	60*	12.8	-16	12.8	1.1	5.86	6.19	
		V 9A:1M	8.6	69*	8.7	+15	+15.2	2.8	3.9	4.6	
	D	VI 9H:1EA	115	+40*	30.4	-15	59.7**	1.3	75.3**	0.1	
		VII 1EA	54	22*	4.5	+23	+23.8	11.2	+41.3**	46	
<i>Ceiba pentandra</i>	H	I 8H:2EA	520	34*	8.9	+5	59.6**	1.9	88.3**	11.6	
		II 6H:4EA	310	46	9.5	+15	20.2	14.8	2.13	26.4	
	D	III 8EA:2M	370	24*	6.1	+20	51.9**	2.7	78.8**	0.39	

H = hexane. D= dichlorometane. EA = ethyl acetate. M = methanol. VN = violacein. GW = growth. PY = pyocyanin. AP = alkaline protease. sd = standar deviation. Yield = mg for every Kg. * $P < 0.05$ by Student's t-test. ** $P < 0.05$ by Bonferroni test.

a homogeneous tissue (nonstratified phellem) composed of numerous cell layers of phellem elongated radially (Figure 3F,G). Furthermore, *C. pentandra* shows some of multiseriate rays strongly dilated near the vascular cambium (wedge-shaped) alternating with irregularly dilated rays (Figure 3F). In contrast, *C. aesculifolia* has multiseriate rays that are longer and irregularly dilated toward the periphery (Figure 3A). Interspersed among the rays, there are evident groups of sclereid cells, which are large and tangentially elongated, in the nonconducting phloem of *C. pentandra*, while those of *C. aesculifolia* are irregularly rounded, smaller and close to periderm (Figure 3C). In the conducting phloem, narrow fiber bands are evident (Figure 3I, J), prismatic crystals are numerous and druses scarce in *C. pentandra* (Figure 3I), while in that of *C. aesculifolia* druses are more numerous (Figure 3E).

Discussion

The bacteria-plant interaction is a phenomenon that has enabled plants to perfect evolutionary strategies, which include production of metabolites to regulate bacterial QSS (Nazzaro *et al.* 2013). *Ceiba aesculifolia* and *C. pentandra* exhibit this type of strategy. Their barks showed QSS regulatory activity, indicating the presence of inhibitor and promoter metabolites. Differ-

Table 2. Results of phytochemical screening of active fractions of *Ceiba aesculifolia* and *Ceiba pentandra*.

Metabolites	Test/reagent	Fraction/Result			
		ICaHex	VICaD	ICpHex	IIICpD
Terpenoids	2 % Vanillin/ 10 % H ₂ SO 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-Burchard	positive	positive	positive	positive
Tannins	FeCl ₃ /Folin-Ciocalteu	negative	negative	negative	negative
Phenols	FeCl ₃ /Folin-Ciocalteu	negative	negative	negative	negative

Ca = *Ceiba aesculifolia*. Cp = *Ceiba pentandra*. Hex = Hexane. D = Dichloromethane.

Figure 2. Dose-response effects of *C. aesculifolia* and *C. pentandra* fractions on production of QSS-controlled virulence factors. A) Growth. B) Alkaline protease activity. Data show the average of at least three independent experiments.

ences in the activity recorded in the two biological systems used may be related to the complexity of the QSS of bacterial species. The two bacterial species used in our study reflect different complexities of their QSS. *C. violaceum* is an aquatic bacterium that can infect humans and cause abscesses and bacteraemia (Stauff & Bassler 2011). The wild-type strain and biosensor mutants of this bacterium are widely used in the study of QSS-I by natural products (Steindler & Venturi 2007) since its purple pigment violacein production is controlled by a single QSS. On the other hand, to date, in *P. aeruginosa* three QS interrelated systems that regulate production of virulence factors such as pyocyanin, pyoverdine, alkaline protease and elastolytic activity have been reported (Christensen *et al.* 2007, Gellatly & Hancock 2013). This bacterium is an opportunistic pathogen that is a major health problem worldwide, responsible for 10 % of nosocomial infections (Antunes *et al.* 2010, Castillo-Juarez *et al.* 2015, García-Contreras 2016) and classified as a pathogen of critical priority by the World Health Organization (WHO 2017).

Figure 3. Sections of *Ceiba* barks: A-J) *C. aesculifolia* transverse sections: B) Stratification of prickles, C) sclereid groups, D) conducting phloem, E) Druses in conducting phloem. F-J) *C. pentandra* transverse sections: G) nonstratified prickles, H) sclereid groups, I, J) conducting phloem. Abbreviations: cp = conducting phloem; f = fibers; np = nonconducting phloem; per = periderm; r = ray; st = sieve tube; sc = stone cells; + = druses; * = prismatic crystal.

Our results indicate that the diversity of metabolites in the two *Ceiba* species is complex. The bark extract exhibited overall QSS-I effect. However, when extracts were fractionated, different effects were found. Several fractions stimulated while others inhibited virulence factors. Moreover, some had a slight effect on strain growth. Regulatory behavior over QSS (positive or negative) may be related to changes in concentration of metabolites as well as to selectivity of the molecules over each QSS. It is necessary to investigate the effect of purified and identified molecules from the extracts to define their selectivity and antagonistic effects on other bacterial QSS, as well as their mechanisms of action.

Although our study did not identify the molecules involved, we identified the major groups of metabolites in the active fractions against the *P. aeruginosa*. Our analysis revealed that they were composed mainly of terpenes, triterpenes and sterols. The active fractions may be an important source of new inhibitor metabolites, potentially expanding the repertoire of QSS-I molecules.

This result is important because there are few reports of this type of metabolites as QSS-I.

However, pentacyclic triterpenes derivatives (oleanane, corosolic, asiatic and ursane) have shown anti-biofilm and anti-virulence activity 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 species of the genus *Dalbergia* showed inhibitory activity against virulence factors of *P. aeruginosa* (Rasamiravaka *et al.* 2013).

Our results suggest the presence of bactericidal molecules in the extracts or fractions, that may mask QSS-I activity. A representative example was the *C. aesculifolia* extract, which reduced the violacein production, but affected bacterial viability. However, presence of bactericidal molecules could complement QSS-I molecules to provide a more potent anti-virulence effect, as demonstrated when asiatic and corosolic acid increased susceptibility of *P. aeruginosa* biofilm to tobramycin (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), favoring the use of lower doses and avoiding indiscriminate use of broad-spectrum antibiotics (Bjarnsholt & Givskov 2007).

As we have seen, the QSS-I activity of the fractions of the extracts of the different *Ceiba* species were not the same, and thus it is important to distinguish between the species. In this sense, the anatomical characteristics of bark may be helpful in species identification (SEMARNAT 2013). Some general traits of bark anatomy of the *Ceiba* genus has been mentioned by Roth (1981), but species-specific traits have not been studied until now. The two species described here follow the general pattern of the genus, but we did find differences. The phellem characteristics of the prickles, ray dilation close to vascular cambium, fibers evident in conducting phloem, as well as the position, size and form of sclereid groups, are the most noticeable bark features that distinguish the two species anatomically. We suggest further anatomical studies to provide information related to the recognition of species and location of possible active principles within the plant tissues.

In this study we determined that extracts and fractions obtained from the two species of *Ceiba* are sources of phytochemicals with the ability to regulate positively or negatively the bacterial QSS evaluated. The active fractions are rich in terpenes and sterols, QSS-I metabolites which have been poorly studied in contrast with other groups. Future work will focus on isolation and identification of these metabolites.

Acknowledgment

This work was supported by grants from Scientific Development Projects for Solving National Problems/CONACyT Mexico no. 2015-01-402. N-MC research is supported by the CONACyT PhD grant 376049, I-CJ research is supported by Fideicomiso-COLPOS 167304 and Cátedras-CONACyT program.

Literature cited

Adonizio AL, Downum K, Bennett BC, Mathee K. 2006. Anti-quorum sensing activity of medicinal plants in Southern Florida. *Journal of Ethnopharmacology* **105**: 427-35. DOI: 10.1016/j.jep.2005.11.025.

- Angyalossy V, Pace MR, Evert RF, Marcati CR, Oskolski AA, Terrazas T, Kotina E, Lens F, Mazzoni-Viveiros SC, Angeles G, Machado SR, Crivellaro A, Rao KS, Junikka L, Nikolaeva N, Baas P. 2016. IAWA list of microscopic bark features. *IAWA Journal* **37**: 517-615. DOI: 10.1163/22941932-20160151.
- Anosike CA, Ogili OB, Nwankwo ON, Eze EA. 2012. Phytochemical screening and antimicrobial activity of the petroleum ether, methanol and ethanol extracts of *Ceiba pentandra* stem bark. *Journal of Medicinal Plants Research* **6**: 5743-47. DOI: 10.5897/JMPR12.978.
- Antunes LC, Ferreira RB, Buckner MM, Finlay BB. 2010. Quorum sensing in bacterial virulence. *Microbiology* **156**: 2271-82. DOI: 10.1099/mic.0.038794-0.
- Avendaño A, Casas A, Dávila P, Lira R. 2006. Use forms, management and commercialization of 'pochote' *Ceiba aesculifolia* (H.B. & K.) Britten & Baker f. subsp. *parvifolia* (Rose) P.E. Gibbs & Semir (Bombacaceae) in the Tehuacán Valley, Central Mexico. *Journal of Arid Environments* **67**: 15-35. DOI: 10.1016/j.jaridenv.2006.02.004.
- Avendaño A, Casas A, Dávila P, Lira R. 2009. *In situ* management and patterns of morphological variation of *Ceiba aesculifolia* subsp. *parvifolia* (Bombacaceae) in the Tehuacán-Cuicatlán Valley. *Economic Botany* **63**: 138-151. DOI: 10.1007/s12231-009-9083-6.
- Bjarnsholt T, Givskov M. 2007. Quorum-sensing blockade as a strategy for enhancing host defences against bacterial pathogens. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **362**: 1213-1222. DOI: 10.1098/rstb.2007.2046.
- Canales M, Hernández T, Caballero J, Romo de Vivar A, Avila G, Duran A, Lira R. 2005. Informant consensus factor and antibacterial activity of the medicinal plants used by the people of San Rafael Coxcatlán, Puebla, México. *Journal of Ethnopharmacology* **97**: 429-439. DOI: 10.1016/j.jep.2004.11.013.
- Castillo-Juarez, I, Maeda T, Mandujano-Tinoco EA, Tomas M, Perez-Eretza B, García-Contreras SJ, Wood, TK, García-Contreras R. 2015. Role of quorum sensing in bacterial infections. *World Journal of Clinical Cases* **3**: 575-598. DOI: 10.12998/wjcc.v3.i7.575.
- Castillo-Juárez I, García-Contreras R, Velázquez-Guadarrama N, Soto-Hernández M, Martínez-Vázquez M. 2013. *Amphypterygium adstringens* anacardic acid mixture inhibits quorum sensing-controlled virulence factors of *Chromobacterium violaceum* and *Pseudomonas aeruginosa* *Archives of Medical Research* **44**: 488-494. DOI: 10.1016/j.arcmed.2013.10.004.
- Christensen LD, Moser C, Jensen P Ø, Rasmussen TB, Christophersen L, Kjelleberg S, Kumar N, Høiby N, Givskov M, Bjarnsholt T. 2007. Impact of *Pseudomonas aeruginosa* quorum sensing on biofilm persistence in an *in vivo* intraperitoneal foreign-body infection model. *Microbiology* **153**: 2312-2320. DOI: 10.1099/mic.0.2007/006122-0.
- de Kievit TR. 2009. Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environmental Microbiology* **11**: 279-288. DOI: 10.1111/j.1462-2920.2008.01792.x.
- Eldridge GR. 2005. Compounds, compositions and methods for controlling biofilms and bacterial infections. US Patent WO/2006/031943.
- Espinosa DS, Ocegueda S, Aguilar C, Llorente-Bousquets J. 2008. El Conocimiento Biogeográfico de las especies y su regionalización natural. In: Conabio. 2008. *Capital Natural de México, vol. I. Conocimiento Actual de la Biodiversidad*. Comisión Nacional para el Conocimiento y Uso de la Biodiversidad, México: ISBN: 978-607-7607 03-8
- Fischbach MA, Walsh CT. 2009. Antibiotics for emerging pathogens. *Science* **325**: 1089-1093. DOI: 10.1126/science.1176667.
- Franco BM, Jiménez-Estrada M, Hernández-Hernández AB, Hernández LB, Rosas-López R, Durán A, Rodríguez-Monroy MA, Canales-Martínez M. 2016. Antimicrobial activity of the fiber produced by 'pochote' *Ceiba aesculifolia* subsp. *parvifolia*. *African Journal of Traditional, Complementary and Alternative Medicines* **13**: 44. DOI: 10.4314/ajtcam.v13i3.6.
- García-Contreras R. 2016. Is quorum sensing interference a viable alternative to treat *Pseudomonas aeruginosa* infections?. *Frontiers in Microbiology* **7**: 1454. DOI: 10.3389/fmicb.2016.01454.
- Garo E, Eldridge GR, Goering MG, Pulcini ED, Hamilton MA, Costerton JW, James GA. 2007. Asiatic acid and corosolic acid enhance the susceptibility of *Pseudomonas aeruginosa* biofilms to tobramycin. *Antimicrobial Agents and Chemotherapy* **51**: 1813-1817. DOI: 10.1128/AAC.01037-06.
- Gellatly SL, Hancock RE. 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathogens and Disease* **67**: 159-173. DOI: 10.1111/2049-632X.12033.
- Gibbs P, Semir J. 2003. A taxonomic revision of the genus *Ceiba* Mill. (Bombacaceae). *Anales Del Jardín Botánico de Madrid* **60**: 259-300. DOI: 10.3989/ajbm.2002.v60.i2.92.
- Gilabert M, Marcinkiewicz K, Andujar S, Schiavone M, Arena ME, Bardón A. 2015. Sesqui- and triterpenoids from the liverwort *Lepidozia chordulifera* inhibitors of bacterial biofilm and elastase activity of human pathogenic bacteria. *Phytomedicine* **22**: 77-85. DOI: 10.1016/j.phymed.2014.10.006.

- Harborne JB. 1984. *Phytochemical Methods*. Dordrecht: Springer Netherlands. ISBN-13: 978-0-412-23050-9 e-ISBN-13: 978-94-009-5921-7; DOI: 10.1007/978-94-009-5921-7.
- Howe TR, Iglewski BH. 1984. Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa* in vitro and in a mouse eye model. *Infection and Immunity* **43**: 1058-1063.
- Hu JF, Garo E, Goering MG, Pasmore M, Yoo HD, Esser T, Sestrich J, Cremin PA, Hough GW, Perrone P, Lee YS, Le NT, O'Neil-Johnson M, Costerton JW, Eldridge GR. 2006. Bacterial biofilm inhibitors from *Diospyros dendo*. *Journal of Natural Products* **69**: 118-120. DOI: 10.1021/np049600s.
- Kishore PH, Reddy MV, Gunasekar D, Caux C, Bodo B. 2003. A new naphthoquinone from *Ceiba pentandra*. *Journal of Asian Natural Products Research* **5**: 227-30.
- Koh CL, Choon KS, Wai FY, Tan LY, Krishnan T, Chong YM, Chan KG. 2013. Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors* **13**: 6217-6228. DOI: 10.3390/s130506217.
- Maeda T, Garcia-Contreras R, Pu M, Sheng L, Garcia LR, Tomas M, Wood TK. 2012. Quorum quenching quandary: resistance to antivirulence compounds. *ISME Journal* **6**: 493-501.
- Muñoz-Cazares N, García-Contreras R, Pérez-López M, 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. DOI: 10.5772/63693; ISBN: 978-953-51-2959-2
- Nazzaro F, Fratianni F, Coppola R. 2013. Quorum sensing and phytochemicals. *International Journal of Molecular Sciences* **14**: 12607-12619. DOI: 10.3390/ijms140612607.
- Ngounou FN, Meli AL, Lontsi D, Sondengam BL, Atta-Ur-Rahman, Choudhary MI, Malik S, Akhtar F. 2000. New isoflavones from *Ceiba pentandra*. *Phytochemistry* **54**: 107-110. DOI: 10.1016/S0031-9422(00)00035-2
- Niembro RA, Vázquez TM, Sánchez OS. 2010. Árboles de Veracruz : 100 especies para la reforestación estratégica. Gobierno del Estado de Veracruz. <http://www.sev.gob.mx/servicios/publicaciones/colec_veracruzsigloXXI/ArbolesVeracruz100especies.pdf> (accessed February 2, 2017)
- Noreen Y, el-Seedi H, Perera P, Bohlin L. 1998. Two new isoflavones from *Ceiba pentandra* and their effect on cyclooxygenase-catalyzed prostaglandin biosynthesis. *Journal of Natural Products* **61**: 8-12. DOI: 10.1021/np970198+.
- O'Malley YQ, Reszka KJ, Spitz DR, Denning GM, Britigan BE. 2004. *Pseudomonas aeruginosa* pyocyanin directly oxidizes glutathione and decreases its levels in airway epithelial cells. *AJP: Lung Cellular and Molecular Physiology* **287**: L94-103. DOI: 10.1152/ajplung.00025.2004.
- Orozco J, Rodriguez-Monroy MA, Martinez KE, Flores CM, Jimenez-Estrada M, Duran A, Rosas-Lopez R, Hernandez LB, Canales M. 2013. Evaluation of some medicinal properties of *Ceiba aesculifolia* subsp. *parvifolia*. *Journal of Medicinal Plants Research* **7**: 309-314. DOI: 10.5897/jmpr12.065.
- Osuntokun OT, Ajayi Ayodele O, Adeoye MI, Odufunwa AE. 2017. Assessment of antimicrobial and phytochemical properties of crude leaf and bark extracts of *Ceiba pentandra* on selected clinical isolates found in nigerian teaching hospital. *Journal of Bacteriology & Mycology* **4**: 1-8. DOI: 10.15406/jbmoa.2017.4.00079.
- Pan J, Ren D. 2009. Quorum sensing inhibitors: a patent overview. *Expert Opinion on Therapeutic Patents* **19**: 1581-1601. DOI: 10.1517/13543770903222293.
- Parulekar GT. 2017. Antibacterial and phytochemical analysis of *Ceiba pentandra* (L.) seed extracts. *Journal of Pharmacognosy and Phytochemistry* **6**: 586-589.
- Pennington TD, Sarukhan J. 2005. Árboles tropicales de México : manual para la identificación de las principales especies. México: Universidad Nacional Autónoma de México-Fondo de Cultura Económica. ISBN: 9703216439, 9789703216437
- Rao KV, Sreeramulu K, Gunasekar D, Ramesh D. 1993. Two new sesquiterpene lactones from *Ceiba pentandra*. *Journal of Natural Products* **56**: 2041-2045. DOI: 10.1021/np50102a003
- Rasamiravaka T, Jedrzejowski A, Kiendrebeogo M, Rajaonson S, Randriamampionona D, Rabemanantsoa C, Andriantsimahavandy A, Rasamindrakotroka A, Duez P, El Jaziri M, Vandeputte OM. 2013. Endemic malagasy *Dalbergia* species inhibit quorum sensing in *Pseudomonas aeruginosa* PAO1. *Microbiology* **159**: 924-938. DOI: 10.1099/mic.0.064378-0.
- Rasmussen TB, Givskov M. 2006. Quorum sensing inhibitors: a bargain of effects. *Microbiology* **152**: 895-904. DOI: 10.1099/mic.0.28601-0.
- Refaat J, Desoky SY, Ramadan MA, Kamel MS. 2013. Bombacaceae: a phytochemical review. *Pharmaceutical Biology* **51**: 100-130. DOI: 10.3109/13880209.2012.698286.
- Ren D, Zuo R, Gonzalez Barrios SF, Bedzyk LA, Eldridge GR, Pasmore ME, Wood TK. 2005a. Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. *Applied and Environmental Microbiology* **71**: 4022-4034. DOI: 10.1128/AEM.71.7.4022-4034.2005.
- Ren D, Zuo R, Wood TK. 2005b. Quorum-sensing antagonist (5Z)-4-Bromo-5-(Bromomethylene)-3-Bu-

- tyl-2(5H)-Furanone influences siderophore biosynthesis in *Pseudomonas putida* and *Pseudomonas aeruginosa*. *Applied Microbiology and Biotechnology* **66**: 689-695. DOI: 10.1007/s00253-004-1691-6.
- Rivera-Arce E, Gattuso M, Alvarado R, Zárate E, Agüero J, Feria I, Lozoya X. 2007. Pharmacognostical studies of the plant drug *Mimosae tenuiflorae* cortex. *Journal of Ethnopharmacology* **113**: 400-408. DOI: 10.1016/j.jep.2007.06.023.
- Rosas-Acevedo H, Terrazas T, González-Trujano ME, Guzmán Y, Soto-Hernández M. 2011. Anti-ulcer activity of *Cyrtocarpa procera* analogous to that of *Amphipterygium adstringens*, both assayed on the experimental gastric injury in rats. *Journal of Ethnopharmacology* **134**: 67-73. DOI: 10.1016/j.jep.2010.11.057.
- Roth I. 1981. Structural Patterns of Tropical Barks. In: Encyclopedia of plant anatomy. Berlin: Bornträger. ISBN: 978-3-443-14012-0
- Roy V, Adams BL, Bentley WE. 2011. Developing next generation antimicrobials by intercepting AI-2 mediated quorum sensing. *Enzyme and Microbial Technology* **49**: 113-123. DOI: 10.1016/j.enzmictec.2011.06.001.
- Ruzin SE. 1999. Plant microtechnique and microscopy. New York: Oxford University Press. ISBN-13: 978-0195089561
- SEMARNAT [Secretaria de Medio Ambiente y Recursos Naturales]. 2013. Programa de manejo-Reserva de la Biosfera Tecuacán-Cuicatlán. <http://www.conanp.gob.mx/que_hacemos/pdf/programas_manejo/tehuacan_2013.pdf> (accessed: February 2, 2017)
- Stauff DL, Bassler BL. 2011. Quorum sensing in *Chromobacterium violaceum*: DNA recognition and gene regulation by the CviR Receptor. *Journal of Bacteriology* **193**: 3871-3878. DOI: 10.1128/JB.05125-11.
- Steindler L, Venturi V. 2007. Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett.* **266**: 1-9. DOI: 10.1111/j.1574-6968.2006.00501.x
- Trockenbrodt M. 1990. Survey and discussion of the terminology used in bark anatomy *IAWA Journal*. **11**: 141-166. DOI: 10.1163/22941932-90000511.
- Ueda H, Kaneda N, Kawanishi K, Alves SM, Moriyasu M. 2002. A new isoflavone glycoside from *Ceiba pentandra* (L.) Gaertner. *Chemical & Pharmaceutical Bulletin* **50**: 403-404.
- Valdivia-Correa B, Gómez-Gutiérrez C, Uribe M, Méndez-Sánchez N. 2016. Herbal medicine in Mexico: a cause of hepatotoxicity. A critical review. *International Journal of Molecular Sciences* **17**: 235. DOI: 10.3390/ijms17020235.
- WHO [World Health Organization]. 2017. Antibacterial agents in clinical development. <http://www.who.int/medicines/news/2017/IAU_AntibacterialAgentsClinicalDevelopment_webfinal_2017_09_19.pdf> (accessed January 17, 2018)
- Zhang LH, Dong YH. 2004. Quorum sensing and signal interference: diverse implications. *Molecular Microbiology* **53**: 1563-1571. DOI: 10.1111/j.1365-2958.2004.04234.x.