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

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## 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. (Malváceas).
**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 esteroles 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 adresssed (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, bioluminescense, 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 naphtaquinones, 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

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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 statiscal 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 096°22.690'W). For *C. pentandra*, samples were obtained in the municipality of Acatlán, state of Oaxaca, Mexico (18° 32.677' N and 096° 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 % $H_2SO_4$ 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 ($10^5$ 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 $OD_{600}$ ~0.05 (UV-1800, Shimadzu). Extracts and fractions were then dissolved in DMSO and 5-10 µL added to 5 mL of the cultures with final concentrations of 128 to 500 µg/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 $mM^{-1}$ $cm^{-1}$ (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).

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 × 2 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 µm) 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 gainst *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 µg/mL) significantly inhibited *P. aeruginosa* virulence factors. CaHex decreased elastolytic activity (26.5 %) and CaD inhibited pyocyanin, pyoverdin and elastolyic 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,

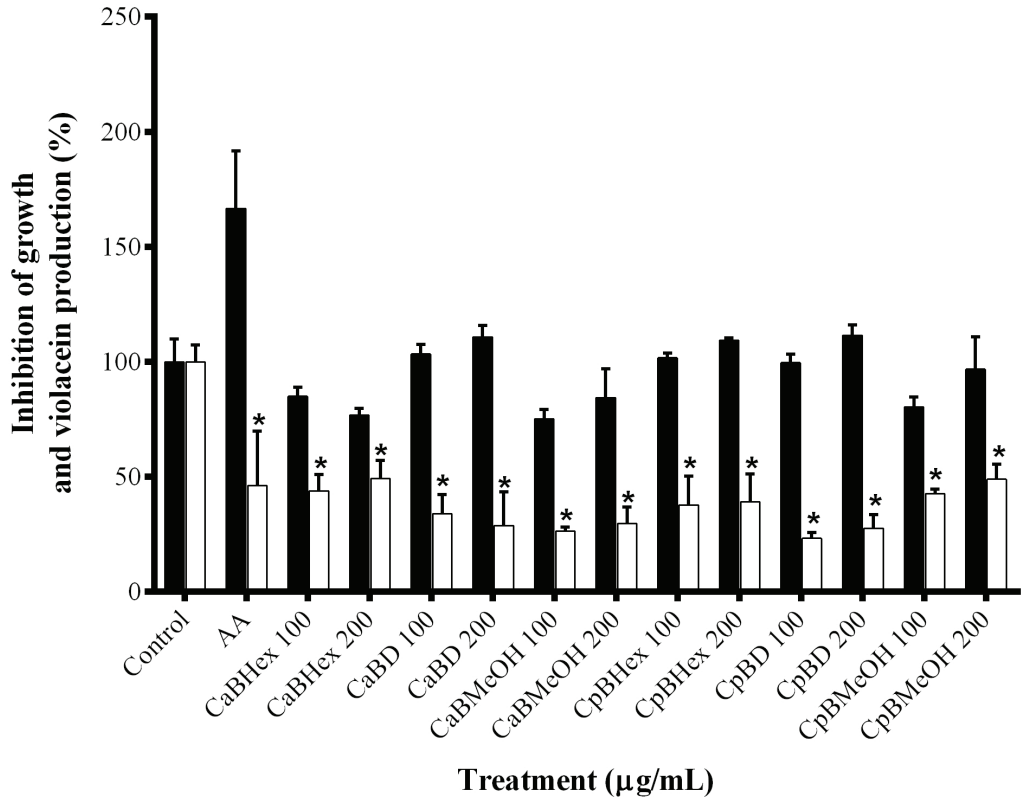


**Figure 1.** Inhibition of violacein production by *C. aesculifolia* and *C. pentandra* bark extracts. Black bars represent bacterial growth (mutant CV026) and white bars represent violacein production (CV12472 WT). AA = anacardic acid mixture. Hex = hexane, D = dichloromethane, MeOH = methanol. **P* < 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 1CpHex.

*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 (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *C. violaceum* (200 µg/mL) | | | *P. aeruginosa* (500 µg/mL) | | | |
| | | | | | VN | *sd* | GW | PY | *sd* | AP | *sd* |
| ***Ceiba aesculifolia*** | 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 |
| ***Ceiba pentandra*** | 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= dichrolometane. 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 % $H_2SO_4$ ethanol | positive | positive | positive | negative |
| Flavonoids | 1 % NP /5 % PEG | negative | positive | positive | negative |
| Alkaloids | Dragendorff | negative | negative | negative | negative |
| Steroids and triterpenoids | Liebermann-Burchard | positive | positive | positive | positive |
| Tannins | FeCl3/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.

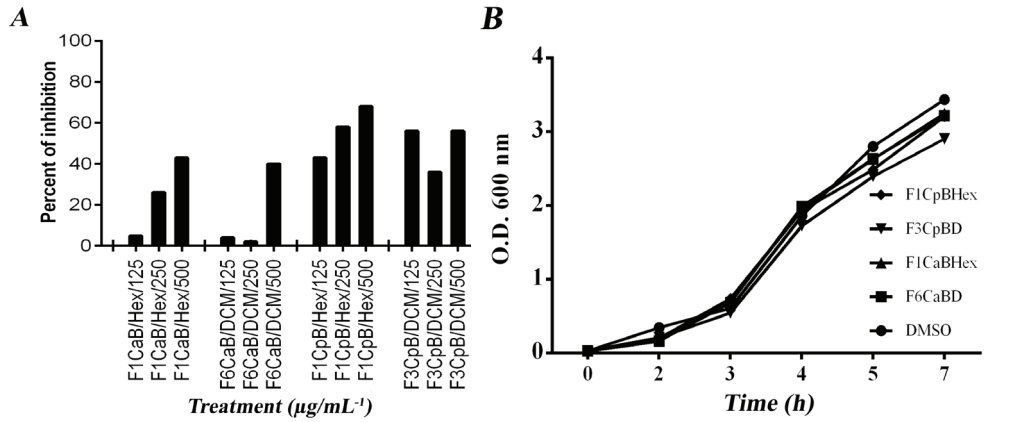


**Figure 2.** Dose-response effects of *C. aesculifolia* and *C. pentandra* fractions on production of QSS-controlled virulence factors. A) Growth. B) Alkaline protease activity. Data show the average of at least three independent experiments.

ences in the activity recorded in the two biological systems used may be ralated 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 prority by the World Health Organization (WHO 2017).

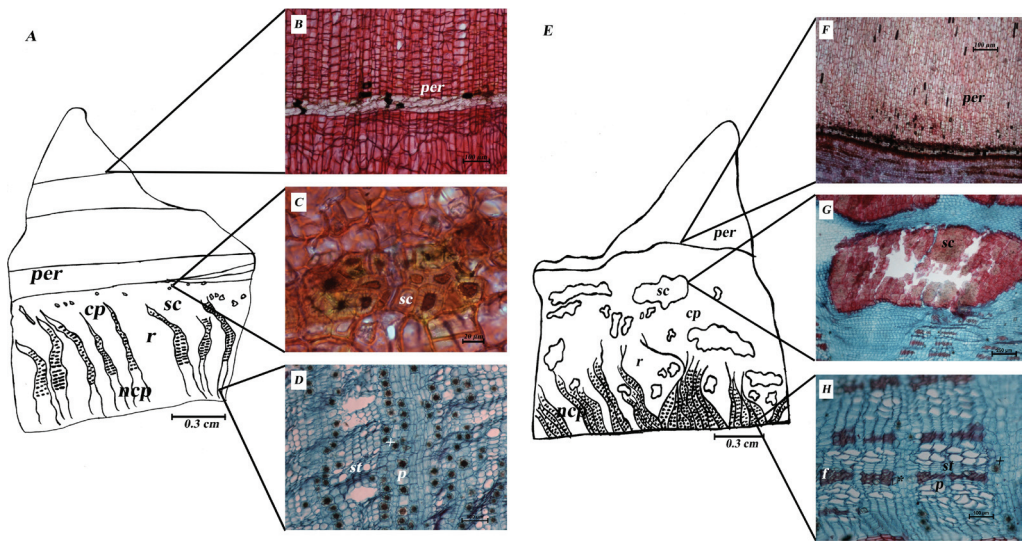


**Figure 3.** Sections of *Ceiba* barks: A-J) *C. aesculifolia* transverse sections: B) Stratification of prickle phellem, C) sclereid groups, D) conducting phloem, E) Druses in conducting phloem. F-J) *C. pentandra* transverse sections: G) nonstratified prickle phellem, H) sclereid groups, I,J) conducting 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 studies 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.

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