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Anti-virulence activities of some *Tillandsia* species (Bromeliaceae)

Actividades antivirulencia de algunas especies de *Tillandsia* (Bromeliaceae)

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Abstract

Background: Using molecules that inhibit bacterial virulence is a potential strategy to fight infections, with the advantage that, in contrast to bactericidal compounds, they do not induce resistance. Several compounds with anti-virulence properties have been identified in plants, however, they represent a small portion of the total diversity, and in Mexico there are still few studies on this matter.
Hypothesis: Extracts of species of the *Tillandsia* genus inhibit the expression of diverse virulence factors without affecting the bacterial growth.
Studied species: *Tillandsia recurvata* (L.) L., *T. schiedeana* Steud. and *T. fasciculata* Sw.
Study site and date: These species were collected in December 2016 in the municipalities of Ixtlahuaca and Santo Tomás de los Plátanos, State of Mexico.
Methods: The ability of dichloromethane (CH_2Cl_2) and methanol (CH_3OH) extracts to inhibit production of violacein in *Chromobacterium violaceum* was evaluated, as well as the virulence factors regulated by quorum sensing, motility and biofilm in *Pseudomonas aeruginosa*. In addition, the bioactive fractions obtained were partially identified by ^{1}H NMR.
Results: CH_2Cl_2 and CH_3OH extracts reduced violacein production from 43 to 85 %, but only those from CH_2Cl_2 reduced protease activity, biofilm formation and swarming. Interestingly, CH_3OH extracts stimulated the formation of biofilms by up to 37 %. Presence of terpenes and phenolic compounds in these species was confirmed. In *T. schiedeana* glycosylated compounds and cycloartane-type triterpenes were identified.
Conclusion: The species of *Tillandsia* show anti-virulence activity, mainly on factors related to adhesion and dispersion in *Pseudomonas aeruginosa*.
Key words: Anti-biofilm, anti-virulence, bromeliads, quorum quenching, *Tillandsia*.

Resumen

Antecedentes: Usar moléculas que inhiben la virulencia bacteriana es una estrategia potencial para combatir infecciones, con la ventaja que, a diferencia de los compuestos bactericidas, no inducen resistencia. Se han identificado en plantas diversos compuestos con propiedades antivirulencia, no obstante, representan una pequeña porción de la diversidad total, además en México son aún escasos los estudios al respecto.
Hipótesis: Los extractos de especies *Tillandsia* inhiben la expresión de factores de virulencia sin afectar el crecimiento bacteriano.
Especies de estudio: *Tillandsia recurvata* (L.) L., *T. schiedeana* Steud. y *T. fasciculata* Sw.
Sitio y año de estudio: Las especies se colectaron en diciembre de 2016 en los municipios de Ixtlahuaca y Santo Tomás de los Plátanos, Estado de México.
Métodos: Se evaluó la capacidad de extractos de diclorometano (CH_2Cl_2) y metanol (CH_3OH) para inhibir la producción de violaceína en *Chromobacterium violaceum*, así como factores de virulencia regulados por percepción de quórum, motilidad y biopelícula en *Pseudomonas aeruginosa*. Las fracciones bioactivas obtenidas se identificaron parcialmente por RMN de ^{1}H.
Resultados: Ambos extractos redujeron la producción de violaceína del 43 a 85 %, pero solo los de CH_2Cl_2 redujeron la actividad proteolítica, formación de biopelícula y motilidad. De modo interesante los extractos de CH_3OH estimularon la formación de biopelícula hasta un 37 %. Se confirmó la presencia de terpenos y compuestos fenólicos en estas especies. En *T. schiedeana* se identificaron compuestos glicosilados y triterpenos tipo cicloartano.
Conclusión: Las especies de *Tillandsia* muestran actividad antivirulencia, principalmente sobre factores relacionados con la adhesión y dispersión en *Pseudomonas aeruginosa*.
Palabras clave: Antibiopelícula, antivirulencia, bromelias, apagado de quórum, *Tillandsia*.

Antibiotics are one of the major discoveries of the 20th century, but their excessive use has generated a rapid development of resistance to this class of drugs (López-Jácome *et al.* 2019). Currently, resistance to antimicrobials is a global public health problem, hence, the WHO has urged to develop new effective therapies against antimicrobial resistant bacteria (Ferri *et al.* 2017). Identification of new antimicrobial action mechanisms that do not induce resistance is an option that has been raised in recent years (Tillotson & Theriault 2013). One of them is anti-virulence therapies, which seek to interfere in the production of virulence factors that bacteria use to establish themselves and cause harm (Muñoz-Cazares *et al.* 2018). The novelty of these therapies is that they do not directly affect the viability of bacteria (as do antibiotics) because their target is a system or metabolic pathway considered non-vital for the bacterial cell (Mühlen & Dersch 2016). To date, different targets that reduce virulence and damage to the host when they are blocked have been identified (Castillo-Juárez *et al.* 2017).

Of the most studied targets are quorum sensing (QS), biofilm formation, type 3 secretion systems (T3SS) and swarming (Muñoz-Cazares *et al.* 2018). QS is a complex phenomenon designed to promote multicellular behavior of unicellular organisms, for which population-level coordination in time and space is required for the expression of virulence factors (Muñoz-Cazares *et al.* 2017). Biofilms are microbial aggregates that allow bacteria to protect themselves from environmental changes, which include tolerance to high doses of antimicrobials (Muñoz-Cazares *et al.* 2018). Similarly, swarming is a social phenomenon that involves rapid coordinated movement by flagella and type IV pili of bacteria on a semisolid surface (Köhler *et al.* 2000).

Chromobacterium violaceum and *Pseudomonas aeruginosa* are the main bacterial models that have been used to identify anti-virulence activity (Castillo-Juárez *et al.* 2013). *C. violaceum* is an opportunistic pathogen of animals that regulates the production of violacein by a QS system (Montes de Oca-Mejía *et al.* 2015). The facility with which QS inhibition is determined through observation of pigment production has made this bacterium one the main biosensors for quorum quenching (Castillo-Juárez *et al.* 2013). *P. aeruginosa* is an opportunistic pathogen of animals and plants and one of the main causes of nosocomial infections. This bacterium regulates the production of virulence factors such as pigments, toxins, enzymes, biofilm formation and swarming through QS. It possesses at least three hierarchically organized QS systems that coordinate production of these virulence factors; hence their inhibition is more complex (Castillo-Juárez *et al.* 2017).

Natural products derived from plants are so far the main source of the largest number of metabolites with anti-virulence properties (Silva *et al.* 2016, Chandra *et al.* 2017). However, the number of species investigated remains minimal in contrast to the enormous diversity of existing plants.

The Bromeliaceae family is composed of 58 genera and 3,408 species native to America distributed from Argentina to the southern United States (Benzing 2000, Luther 2014). Some species have important pharmacological activities, such as anthelmintic (Stepek *et al.* 2005), antinociceptive (de Lima-Saraiva *et al.* 2014), gastroprotective (Machado *et al.* 2013), photoprotective (de Oliveira-Júnior *et al.* 2017), anticancer (Lowe *et al.* 2017), hypoglycemic (Witherup *et al.* 1995) and antibacterial (Faller *et al.* 2017) activity.

In Mexico, there are19 genera and 422 species of bromeliads, of which 230 species correspond to the *Tillandsia* genus, one of the most diverse genera in our country (Espejo-Serna & López-Ferrari 2018). This genus includes species that are mainly ornamental and medicinal (Mondragón-Chaparro *et al.* 2011). In traditional medicine, they are used to treat infections, coughs, bronchitis, burns and gastritis (Sandoval-Bucio *et al.* 2004). Moreover, their bactericidal activity, mainly against Gram-positive bacteria, has been documented (Castillo-Juárez *et al.* 2009, Vite-Posadas *et al.* 2011, Silva *et al.* 2013). Interestingly, their anti-virulence properties have not been investigated, although the chemical composition of this family is characterized by the presence of compounds identified in other species as possessing the ability to reduce virulence, such as flavonoids, sterols, cinnamic acid derivatives and lignans (Manetti *et al.* 2009, Silva *et al.* 2016).

Therefore, the objective of this research was to analyze the violacein inhibition and anti-virulence potential of three species of the *Tillandsia* genus (*T. recurvata* (L.) L., *T. schiedeana* Steud. and *T. fasciculata* Sw.) distributed in Mexico and their effect on the inhibition of virulence factors in *P. aeruginosa*.

Materials and methods

Plant material. Plants were collected in December 2016 in the west-central area of the State of Mexico. *T. recurvata* and *T. schiedeana* were collected in "La Pedrera", Ixtlahuaca (19° 10. 662' N; 100° 15.303' W), altitude 1,368 m asl, and *T. fasciculata* was collected in "El Pedregal", Santo Tomás de los Plátanos (19° 11.167' N; 100° 15.912' W), altitude 1,216 m asl. The specimens were identified by Dr. María Flores Cruz (Figure 1 A-C) and deposited in the Collection of Living Bromeliads at the University Center for Conservation and Research of Mexican Bromeliads (CUCIBROM).

Extract preparation. The dried and powdered tissues of the whole plant (10 g) were de-fatted with 200 mL of hexane (JT Baker). They were extracted sequentially with similar

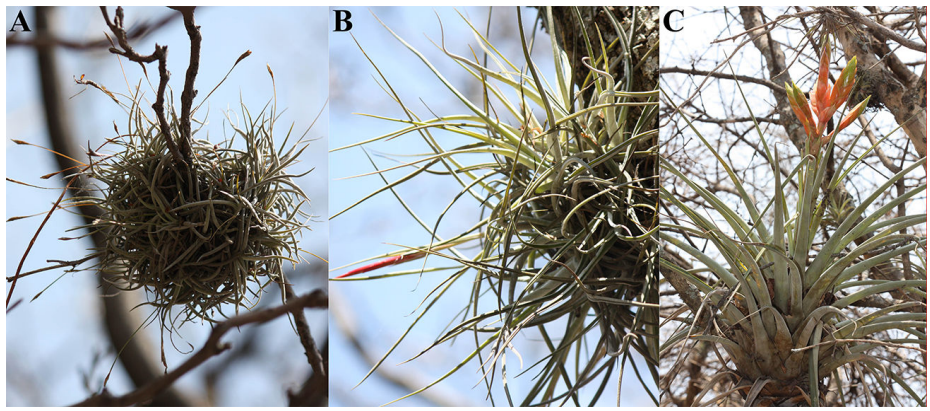

Figure 1. A: *Tillandsia recurvata,* B: *T. schiedeana* and C: *T. fasciculata*. Photographs taken in the municipalities of Ixtlahuaca and Santo Tomás de los Plátanos, State of Mexico.

volumes of dichloromethane (JT Baker) and methanol (JT Baker), evaporated under reduced pressure (Buchi-R114, Switzerland) and kept refrigerated at 4 °C. Organic extracts were subjected to chemical testing following the methods described by Soto-Hernández *et al*. (2019) for identification of terpenes, flavonoids, phenols and tannins.

Fractionation and chemical identification. The samples of *T. recurvata* (9.39 g) and *T. schiedeana* (200 g) were sequentially extracted (1:2 w/v) with hexane (to defatted, × 1), dichloromethane (D, × 3) and methanol (MeOH, × 3). Evaporation was carried out with a rotary evaporator under low pressure, yielding *T. recurvata*-DCM (Tr-D, 131 mg), *T. recurvata*-MeOH (Tr-MeOH, 488 mg), *T. schiedeana*-DCM (Ts-D, 2.79 g) and *T. schiedeana*-MeOH (Ts-MeOH 14.79 g) of crude extracts.

The fractionation of the extracts was performed by low pressure column chromatography (silica gel 60, 70-230 mesh, Merck®) with increasing polarities of hexane and ethyl acetate (J.T. Baker®) (Figure S1 and S2). In Tr-D, 1.6 mg of a solid (Tr-D4) was obtained, while in Tr-MeOH, 2.5 mg (Tr-MeOH3) and 5 mg (Tr-MeOH6) of other compounds were precipitated.

In Ts-D, 37 fractions were obtained, which were grouped into four final fractions according to their thin layer chromatographic profile: Ts-D13-15 (13.3 mg), Ts-D18-23 (15 mg), Ts-D33-35 (1.3 mg) and Ts-D36-37 (1.3 mg). In Ts-MeOH, the mother liquor was separated by low-pressure column chromatography (silica gel 60, 70-230 mesh, Merck®), obtaining 61 fractions, from which it was possible to isolate Ts-MeOH57-61 (5.8 mg), Ts-MeOH31 (2.1 mg), Ts-MeOH11 (traces) and Ts-MeOH9 (21.5 mg). In the case of *T. fasciculata*, the biological material available was not sufficient to carry out a fractionation.

Finally, the identification by physical methods was performed by ^{1}H NMR, using a Bruker-Avance 300 spectrometer (300 MHz), and dissolving each sample in $CDCl_3$. Tetramethylsilane (TMS) was used as internal reference. Chemical shifts were expressed in parts per million (ppm) and coupling constants (*J*) were reported in Hz.

Inhibition of violacein production. Overnight cultures of *C. violaceum* ATCC 553 were adjusted to OD_{620nm} = 0.1 (Dynamica Halo MPR-96), and 200 µL per condition were added in a 96-well microplate (Corning® Costar). The extracts were dissolved in dimethyl sulfoxide (DMSO, Merck) and 5µL was added to obtain final concentrations of 62.5, 125, 250 and 500 µg mL^{-1}. Subsequently, they were incubated at 28 °C, 150 rpm. DMSO was used as a negative control and a mixture of anacardic acids (AA) as a positive control (Castillo-Juárez *et al.* 2013).

A plate count was performed to determine the effect on bacterial growth at 48 h. The determination of violacein production was carried out according to Castillo-Juárez *et al.* (2013). The pigment was extracted with ethyl acetate (J.T. Baker) and evaporated at room temperature for 24 h. The dried violacein was solubilized in 200 µL of 80 % ethanol and quantified at 620 nm. Experiments were performed in triplicate. The results were converted to percentage of violacein produced with respect to the untreated control (DMSO).

Inhibition of QS-regulated factors in P. aeruginosa. Overnight cultures of *P. aeruginosa* PA14 strain (37 °C, 200 rpm) were adjusted to $OD_{600\ nm}$ = 0.05 in 5 mL of LB medium (Spectronic® Genesys 5) and incubated until the turbidity reached OD_{600nm} = 1.0, and then when 62.5µL of each sample was added to obtain a final concentration of 250 µg mL^{-1}. The cultures were incubated for 6 h more and production of virulence factors and growth at 600 nm was quantified.

Pyocyanin production was determined as follows: cultures were centrifuged at 13,000 rpm for 5 minutes. To the supernatant (800 µL) of each culture, 450 µL of chloroform was added and vigorously stirred for 2 minutes.

It was again centrifuged to recover the organic phase, to which 800 µL of 0.2 N hydrochloric acid was added. Subsequently, 650 µL of the aqueous phase was separated and the same volume of water was added. Finally, absorbance was measured at 520 nm (Spectronic® Genesys 5). Bacterial growth was measured at 600 nm. DMSO was used as the negative control, and production of pyocyanin was normalized by growth.

Elastolytic activity in the supernatants was measured by the Elastin-Congo red (ECr) (Sigma Aldrich) assay. Five milligrams ECr was mixed with 950 µL buffer (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5) and 50 µL of the supernatant previously diluted with buffer (1:10, v/v). This mixture was incubated 2 h (37 °C at 200 rpm) and centrifuged for 5 minutes at 13,000 rpm. Absorbance of the supernatant was measured at 495 nm (Spectronic® Genesys 5). At each value, the absorbance of the control was subtracted (LB medium diluted 1:10). Activity was expressed in 495 nm absorbance/growth (600 nm).

Alkaline exoprotease production was determined using Hide-Remazol Brilliant Blue R (HRBBR) (Sigma Aldrich) as substrate. To 875 µL of reaction buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0) were added to 125 µL of bacterial culture supernatant (diluted 1:10 in buffer) and 5 mg of HRBBR. The mixture was incubated (35 minutes at 37 °C and 200 rpm) and was centrifuged at 13,000 rpm for 5 minutes to remove the unhydrolyzed portion. Absorbance was determined at 595 nm (Spectronic® Genesys 5).

Biofilm formation was evaluated by microtiter plate method. Overnight culture (15 h at 37 °C, 200 rpm) of PA14 was adjusted to an OD_{600nm} = 0.05 (Spectronic® Genesys 5) and 200 µL per sample was added in 96-well plates (Corning® Costar). Subsequently, 5 µL of the extracts were incorporated to obtain concentrations of 62.5, 125, 250 and 500 µg mL^{-1}. DMSO was used as negative control and furanone C-30 at 50 µM was a positive control. The mixture was incubated without shaking for 24 h at room temperature. The biofilm adhered to the plate was stained with 200 µL of crystal violet (0.1 %, w/v) and washed three times with distilled water. The adhered dye was solubilized with 80 % ethanol and absorbance was measured at 570 nm (Thermo Scientific).

Soft-agar motility assay was performed in order to examine swarming motility of bacteria. Extracts (250 and 500 µg mL^{-1}) were added to M8 minimal medium supplemented with 0.2 % glucose, 0.5 % casamino acids, 1 mM $MgSO_4$ and 0.5 % agar (Ha *et al.* 2014). The assay was performed in 6-well plates that were inoculated with 2.5 µL of an overnight culture of PA14 (12 h). After 12 h of incubation at 37 °C (Riossa E-71 culture oven), motility diameters were measured, and the results were transformed to swarming percentage with respect to the control (DMSO). The assay was performed with three independent cultures.

Statistical analysis. Data were processed with SigmaPlot software version 10 (Systat Software, Inc., San Jose California USA). Violacein production and biofilm formation were analyzed through ANOVA and the Dunnett test ($\alpha = 0.05$) to compare the means of the treatments with DMSO (control). Pyocyanin, protease, elastase production and motility were compared using the Student´s t-test ($P \leq 0.05$). Both tests were performed in the Statistic software V.9 Analytical Software, Tallahassee, FL, USA.

Results

Quorum sensing inhibition in Chromobacterium violaceum. The organic extracts of the three species reduced violacein production without affecting bacterial growth, suggesting that they contain metabolites whit quorum quenching activity. At the lowest dose evaluated (62.5 µg mL^{-1}), the CH_2Cl_2 extracts reduced pigment production by 43 to 52 %, but only *T. recurvata* and *T. schiedeana* were able to inhibit 68 to 76 % at 250 µg mL^{-1} (Figure 2A). This effect is similar to that exhibited by the AA used as positive control.

The CH_3OH extracts showed a dose-response effect. *T. recurvata* at 500 µg mL^{-1} inhibited the production of violacein by 85 %, whereas *T. schiedeana* and *T. fasciculata* inhibited only 58 % to 65 % (Figure 2B).

Quorum sensing inhibition in Pseudomonas aeruginosa. Extracts exhibited a moderate effect on inhibition of QS-regulated virulence factors in this bacterium. Although none inhibited pyocyanin production, the CH_2Cl_2 extracts of the three species reduced alkaline protease activity from 20 to 33 %. *T. recurvata* and *T. schiedeana* also reduced elastase activity by about 23 % (Table 1). It should be mentioned that for the case of CH_3OH extracts that affected elastase inhibition, the effect was not statistically significant (Table 1).

Inhibition of biofilm formation and swarming in Pseudomonas aeruginosa. CH_2Cl_2 extracts of the three species inhibited biofilm formation (32 to 51 %), although this effect was less than that exhibited by the positive control, furanone C-30 (50 µM), which reduced it by 79 % (Figure 3A). On the other hand, the CH_3OH extracts had an opposite effect; they stimulated biofilm formation. *T. schiedeana* (500 µg mL^{-1}) promoted an increase of 37 % (Figure 3B).

Regarding bacterial motility, the CH_2Cl_2 extracts of the three species inhibited *P. aeruginosa* swarming. *T. recurvata* showed the best effect by reducing motility by 50 % at 500 µg mL^{-1}, similar to that shown by furanone C-30 used as the positive control. *T. schiedeana* reduced motility by 28

and 39 %, and *T. fasciculata* did so by 7 and 43 % at the concentrations of 250 and 500 µg mL^{-1}, respectively (Figure 4). There was no effect on inhibition of motility by the CH_3OH extracts.

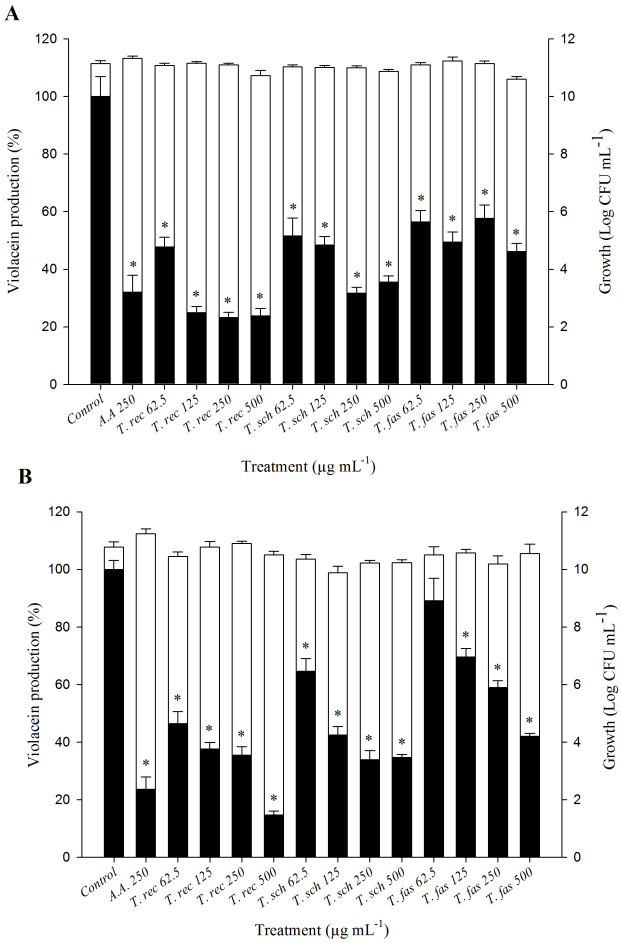

Figure 2. Effect of CH_2Cl_2 (**A**) and CH_3OH (**B**) extracts of *Tillandsia recurvata* (*T. rec*), *T. schiedeana* (*T. sch*) and *T. fasciculata* (*T. fas*) in the violacein production (black bars) and growth (white bars) of *Chromobacterium violaceum*. Black lines represent standard error of the mean. *$P < 0.05$.

Preliminary phytochemical analysis indicated that dichloromethane extracts of the three species contain mainly terpenoid compounds and in a smaller proportion of phenolic type metabolites. In the case of methanol extracts, they mainly contain compounds of a phenolic nature, such as flavonoids and to lesser extent terpenoids. Interestingly, none of the samples contained tannins (Table 2. Figure S3). For the chemical identification of the constituents that conform the extracts, separation was carried out using conventional chromatography techniques and identification by ^{1}H NMR (Table 3).

For the compounds and fractions of *T. recurvata* in Tr-D4, at δ_H 7.6 and 6.5, signals of aromatic protons were identified and attributed to the flavonoid backbone. Similarly, signals between δ_H 12-13 indicate the presence of three distinct phenolic protons at C-5, chelated with the carbonyls at C-4 in the flavonoid framework (Table 3. Figure S4) (VH *et al*. 2014). Compounds of this nature such as 5,3'dihydroxy-6,7,8,4'-tetramethoxyflavanone have been isolated from *T. recurvata* (de Queiroga *et al*. 2004). In Tr-MeOH3, a pentacyclic type-triterpene was identified as the principal constituent of the fraction, which is supported by signals δ_H 5.36, 3.9 and 0.68, 0.82 that are characteristic of this class of molecules (Table 3. Figure S5) (Woo *et al*. 2015). In the Tr-MeOH6 fraction, it was identified as a mixture of methoxylated flavonoids, which is supported by the signals of aromatic protons (δ_H 7.7 to 6.5), that of a C-5 phenolic proton (δ_H 12.19) and that a methoxy group (δ_H 3.98) (Table 3. Figure S6).

Table 1. Anti-virulence effect of CH_2Cl_2 and CH_3OH extracts of three species of the genus *Tillandsia* on the inhibition of QS-regulated virulence factors in *Pseudomonas aeruginosa*.

	% inhibition:	
Specie-extract	**Elastase**	**Protease**
T. recurvata-CH_2Cl_2	23.75±6.05*	28.50± 3.16*
T. schiedeana-CH_2Cl_2	22.43±3.00*	20.98±6.76*
T. fasciculata-CH_2Cl_2	---	33.93±10.61*
T. recurvata-CH_3OH	21.77±4.69	---
T. schiedeana-CH_3OH	24.95± 11.19	---
T. fasciculata-CH_3OH	18.53± 6.34	1.66±5.60

--- No effect, * significant differences ($P < 0.05$). Average ±

For *T. schiedeana*, Ts-D13-15 was identified as a mixture of fatty acids that is supported by the δ_H 1.26 signal (Figure S7). Ts-MeOH31 was identified a compound related to β-sitosterol, which supported by signals attributable to sterols such as δ_H 2.3 and 3.9 (Table 3. Figure S8) (Bulama *et al*. 2015). While in Ts-MeOH11 the characteristic signals of β-sitosterol (δ_H 2.2, 3.5, 5.3) are observed (BMRB 2019), indicating that in this mixture there is a majority presence of this sterol or a compound with a very similar structure (Table 3. Figure S9). In Ts-MeOH57-61, signals at δ_H 4.5 and 3.2 among others were identified (Table 3. Figure S10), as characteristic of sugar residue protons (Primahana & Darmawan 2017). This indicates the presence of glycosides in both flavonoids (δ_H 7.0-7.5), as well as triterpenes (δ_H 2.5-1.0) (Barbosa *et al*. 2010, Ibrahim *et al*. 2019). Finally, in Ts-MeOH9 the presence of two doublets at δ_H 0.33 ($J = 4.2$ Hz) and at δ_H 0.55 ($J = 4.2$ Hz), is indicative of cyclopropane protons in the cycloartane framework (Table 3. Figure S11) (Maatooq *et al*. 2002). This class of triterpenes has been previously isolated from *T. recurvata*

(Cabrera & Seldes 1995). Likewise, signals are observed in the aromatic region, indicating that flavonoids are also present in this fraction (Primahana & Darmawan 2017).

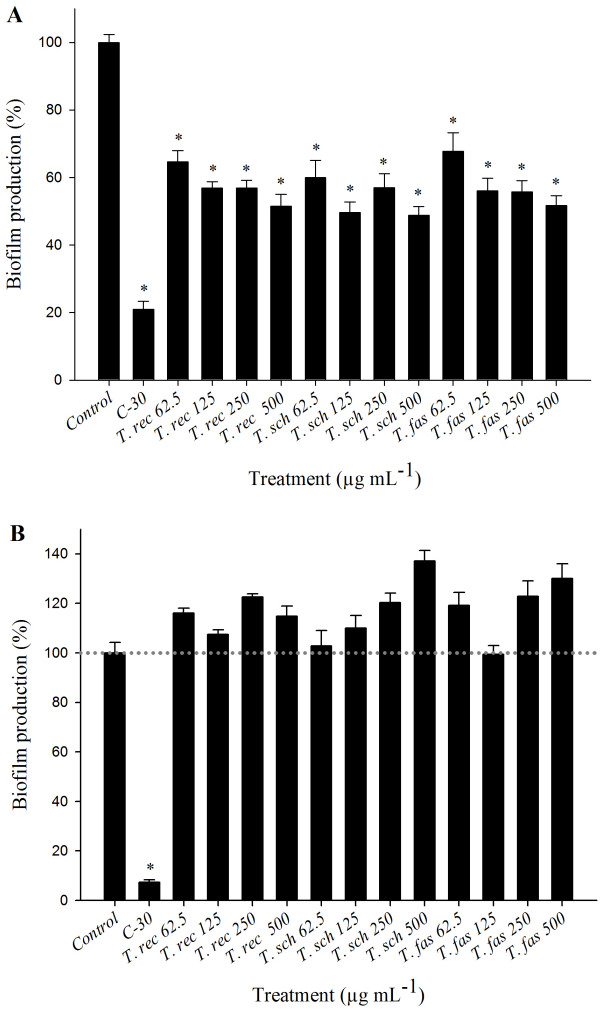

Figure 3. Effect of CH_2Cl_2 (**A**) and CH_3OH (**B**) extracts of *Tillandsia recurvata* (*T. rec*), *T. schiedeana* (*T. sch*) and *T. fasciculata* (*T. fas*) in biofilms formation of *Pseudomonas aeruginosa*. Black lines represent standard error of the mean. **P* < 0.05.

Discussion

Natural products are the main source of antimicrobial compounds. Specifically, those produced by microorganisms have been the main suppliers of the antibiotics that are currently used in the clinic (Bérdy 2012). However, the constant emergence of resistance has decreased effectiveness of antibiotics and has compromised their use against antibiotic-resistant strains (López-Jácome *et al.* 2019). Faced with this situation, anti-virulence therapies have been proposed for treatment of bacterial infections, without inducing the appearance of resistance since they do not directly affect viability of microorganisms (Castillo-Juárez *et al.* 2017). This class of molecules acts by inhibiting the production of virulence factors that participate in the establishment and generation of damage in infectious processes (Muñoz-Cazares *et al.* 2017). However, although its use is feasible, up to now the low efficiency of the molecules in *in vivo* assays and the lack of clinical studies have limited its application (García-Contreras 2016). Moreover, canonical quorum quenching compounds such as furanone C-30 and 5-fluorouracil are highly toxic and are not effective against several clinical strains (García-Contreras *et al.* 2015, Guendouze *et al.* 2017).

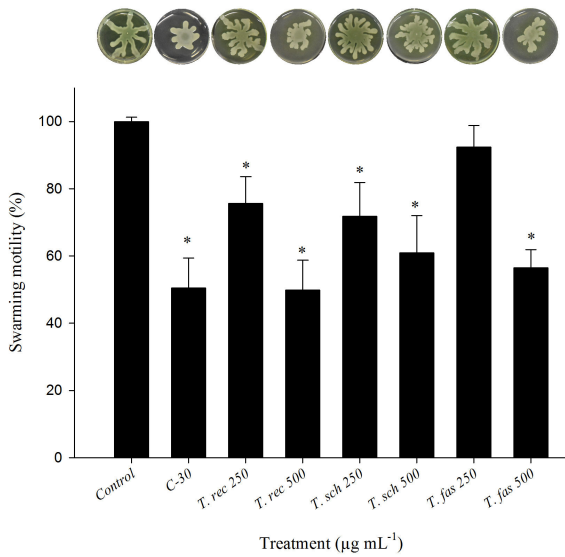

Figure 4. Effect of CH_2Cl_2 extract of *Tillandsia recurvata* (*T. rec*), *T. schiedeana* (*T. sch*) and *T. fasciculata* (*T. fas*) in swarming of *Pseudomonas aeruginosa*. Black lines represent standard error of the mean. **P* < 0.05.

Due to the growing problem of resistance to antibiotics, plants have become an important source in the search for metabolites with mechanisms of action that are not bactericidal (Silva *et al.* 2016). In this respect, the greatest number of molecules capable of inhibiting virulence, mainly QS and biofilm formation, have been identified in plant species (Ta & Arnason 2015, Silva *et al.* 2016). It should be noted that they are not new chemical structures, and they can be found in different genera of plants distributed in different parts of the world. Some reports have focused on investigating the anti-virulence properties of local species or of traditional remedies, but in Mexico studies are still scarce. In this work, we focused on investigating the anti-virulence properties of three species of *Tillandsia* distributed in Mexico as potential source of this class of molecules.

Table 2. Preliminary phytochemical screening of *Tillandsia* species

Metabolites	Reagent	Species	Extract	
			CH_2Cl_2	CH_3OH
Terpenoids	$CHCl_3$, acetic anhydride, H_2SO_4	*T. recurvata*	+++	+
		T schiedeana	+++	+
		T. fasciculata	+++	-
Phenols	Na_2CO_3 2.5 %, Folin-Ciocalteu 10 %	*T. recurvata*	+++	+++
		T schiedeana	++	+++
		T. fasciculata	+	+++
Flavonoids	Mg, HCl	*T. recurvata*	-	++
		T schiedeana	-	++
		T. fasciculata	-	++
Tannins	$FeCl_3$ 5 %- TLC	*T. recurvata*	-	-
		T schiedeana	-	-
		T. fasciculata-	-	-

High (+++), medium (++), low (+) and null (-). TLC = thin layer chromatography

Table 3. Main chemical compounds identified in *Tillandsia* species

Organic extract	Fraction (mg)	^{1} H NMR spectral data (δ, ppm, 300 MHz)	Compounds
T. recurvata-dichloromethane	Tr-D4 (1.6)	13.0-12.0, 7.6, 6.5,	Possible: 5,3´dihidroxi-6,7,8,4´-tetrametoxiflavanone
T. recurvata- MeOH	Tr-MeOH3 (2.5)	5.36, 3.9, 0.68, 0.82	Pentacyclic type-triterpene
	Tr-MeOH6 (5)	12.2, 7.7-6.5, 3.98	Methoxylated flavonoids mixture
T. schiedeana-dichloromethane	Ts-D13-15 (13.3)	1.26	Fatty acids mixture
T. schiedeana-MeOH	Ts-MeOH31 (2.1)	3.9, 2.3	Related to sterol
	Ts-MeOH11	5.3, 3.5, 2.2	β-Sitosterol
	Ts-MeOH57-61 (5.8)	7.5-7-0, 4.5, 3.2, 2.5-1.0	Flavonoids or triterpenes glycosylated
	Ts-MeOH-9 (21.5)	0.55 (J = 4.2 Hz) and 0.33 (J = 4.2 Hz)	Cycloartane-type triterpenes mixture

Chromobacterium violaceum is one of the main biosensors used to evaluate QS inhibition because it produces a violet pigment which facilitates visualizing the effect on the QS inhibition, which is in relation to the amount of pigment inhibited (McClean *et al.* 1997). Although generally *Tillandsia* extracts do not completely prevent violacein production, this bioassay suggests that these species contain metabolites that potentially inhibit QS. This effect is similar to other reported extracts, which do not completely inhibit pigment production and potentiate their activity only with purified active compounds (Castillo-Juárez *et al.* 2013). On the other hand, the only study that has been carried out on the anti-virulence potential of these species was conducted with ethanolic and aqueous extracts of *T. recurvata*, which did not reduce violacein production (Adonizio *et al.* 2006). Because we identify activity in extracts of lower polarity, the active compounds may not be extracted in large quantities with ethanol or water.

Pseudomonas aeruginosa QS systems have also been extensively investigated. This microorganism contains at least three identified systems that are regulated hierarchically to produce several virulence factors, such as pigments (pyocyanin and pyoverdine), enzymes (protease and elastase), swarming and biofilms (Lee & Zhang 2015). It has been reported that the aqueous extract of *Ananas comosus* (Bromeliaceae) inhibits proteolytic and elastolytic activity, as well as biofilm formation and, partially, pyocyanin production (Musthafa *et al.* 2010). However, the

extracts of the three species of *Tillandsia* evaluated had no effect on pyocyanin production and only the CH_2Cl_2 extract slightly reduced protease and elastase activity. Similarly, swarming and biofilm formation decreased with less polar extracts. Interestingly, when the extract is more polar, as in the case of CH_3OH, the inhibitory activity on swarming is lost and biofilm formation is stimulated. In this regard, it has been reported that different molecules with capacity to inhibit or stimulate QS can coexist in a plant extract.

In addition, it has been reported that certain phenolic compounds inhibit virulence factors, while others stimulate their production, depending on their concentration (Plyuta *et al.* 2013). Similarly, in the case of some essential oils, structural isomerism is also important for the oil to stimulate or inhibit the violacein and pyocyanin production (Ahmad *et al.* 2015). Varposhti *et al.* (2013) indicates that *Dianthus orientalis* and *Origanun majorana* extracts also increase biofilm production depending on the type of extraction. On the other hand, stimulation of biofilm cannot be considered a negative effect because it has been shown that its stimulation by certain extracts or metabolites induce defective formation, making it more sensitive to destruction by antimicrobial agents.

Chemical composition of the Bromeliaceae family is characterized by the presence of abundant terpenes, flavonoids and phenols (Manetti *et al.* 2009). However, of the species studied, only *T. recurvata* and *T. fasciculata* have been reported to contain some compounds (Estrella-Parra *et al.* 2019). In this study, we corroborate the presence of terpenes, flavonoids and phenols in the three *Tillandsia* species and in the case of *T. schiedeana,* it is the first report describing the kinds of specialized metabolites derived from the species. However, that it was not possible to obtain pure compounds, the analysis of the fractions allowed us to identify various chemical groups such as glycosylated compounds (flavonoids and cycloartane-type triterpenes). Flavonoids isolated from plant species are the main group of molecules that have been identified as having anti-virulence properties on different bacterial species (Silva *et al.* 2016). However, of those reported with this activity for the species analyzed in this study, only quercetin has been identified in *T. fasciculata* (Williams 1978). On the other hand, the low polarity extract of *Tillandsia* has an inhibiting effect on QS-regulated virulence factors of *P. aeruginosa* and represent an important source of anti-virulence molecules to be explored.

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Supplemental data

Supplemental material for this article can be accessed here: https://doi.org/10.17129/botsci.2380

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