VITAE, REVISTA DE LA FACULTAD DE QUÍMICA FARMACÉUTICA
ISSN 0121-4004 / ISSNe 2145-2660. Volumen 18 número 1, año 2011.
Universidad de Antioquia, Medellín, Colombia. págs. 77-82

ANTIQUORUM SENSING ACTIVITY OF ESSENTIAL OILS ISOLATED FROM DIFFERENT SPECIES OF THE GENUS *Piper*

ACTIVIDAD ANTIQUÓRUM SENSING DE ACEITES ESENCIALES AISLADOS DE DIFERENTES ESPECIES DEL GÉNERO *Piper*

Jesús T. OLIVERO V. [1*], Nerlis P. PÁJARO C. [1], Elena STASHENKO [2]

Received: 21 April 2010 Accepted: 16 November 2010

ABSTRACT

Quorum sensing is a bacterial communication mechanism that depends on population density, and occurs through molecules called autoinducers. These molecules activate receptors, enabling the transcription of genes that encode information needed to control several biochemical mechanisms associated with bacterial survival and pathogenicity. The aim of this study is to evaluate the inhibitory effect of essential oils of three species of *Piper* on the production of violacein, induced by *N*-hexanoyl homoserine lactone in *Chromobacterium violaceum* CV026. Results show that essential oils from *Piper bredemeyeri*, *Piper brachypodom* and *Piper bogotence* present 50% inhibitory concentration (IC_{50}) for quorum sensing of 45.6 μg/mL, 93.1 μg/mL, and 513.8 μg/mL, respectively. However, in terms of cell growth, IC_{50} values for these oils are greater than 1000 μg/mL. These data suggest that essential oils isolated from *Piper* species found in Colombian flora are good candidates for the development of antiquorum sensing molecules, with possible applications in the control of bacterial diseases.

Keywords: Inhibition, quorum sensing, essential oils, genus *Piper,* bacterial infections.

RESUMEN

El quórum sensing es un mecanismo de comunicación bacteriana que depende de la población celular y que ocurre a través de moléculas llamadas autoinductores, las cuales activan receptores que permiten la transcripción de genes que codifican la información necesaria para controlar diversos mecanismos bioquímicos asociados con la supervivencia y la patogenicidad bacteriana. El objetivo de este trabajo consiste en evaluar el efecto inhibitorio de los aceites esenciales de tres especies del género *Piper* sobre la producción de violaceína inducible por *N*-hexanoil homoserina lactona en *Chromobacterium violaceum* CV026. Los resultados señalan que los aceites esenciales de *Piper bredemeyeri*, *Piper brachypodom* y *Piper bogotence* poseen una concentración inhibitoria 50% (CI_{50}) para el quórum sensing de 45.6 μg/mL, 93.1 μg/mL y 513.8 μg/mL, respectivamente. Sin embargo, en términos de crecimiento celular, el valor de CI_{50} para estos aceites es mayor de 1000 μg/mL. Estos datos sugieren que los aceites esenciales aislados de especies de *Piper* encontrados en la flora Colombiana son buenos candidatos para el desarrollo de moléculas antiquórum sensing, con posibles aplicaciones en el control de enfermedades bacterianas.

Palabras clave: inhibición, quórum sensing, aceites esenciales, género *Piper,* infecciones bacterianas.

[1] Environmental and Computacional Chemistry Group. Faculty of Pharmaceutical Sciences. Universidad de Cartagena. Campus of Zaragocilla. Cartagena, Colombia.

[2] CENIVAM. Campus UIS. Building C 45. Bucaramanga, Colombia.

* Corresponding author: joliverov@unicartagena.edu.co

INTRODUCTION

Quorum sensing (QS) is a cell communication mechanism through which signal molecules called autoinducers activate specific receptors associated with transcription signals for controlling various biochemical processes. Some of these processes are biofilm formation, expression of virulence factors, luminescence, pigment production, and mechanisms of resistance to stress conditions (2), which are of major importance in bacterial pathogenesis (1-3). For these reasons, the search for antiquorum sensing compounds (Anti-QS) offers a new perspective on the application of natural or pure compounds as therapeutic agents, which by inhibiting this mechanism of cell communication could be used to control bacterial diseases (4).

Nature offers a wide range of plants, algae and bacteria capable of inhibiting QS mechanisms. This fact has allowed the development of various studies aimed at finding new options for treatment of infections caused by pathogenic bacteria (5-9). Accordingly, QS systems are molecular targets with applications in various fields, including medicine, veterinary medicine, agriculture and aquaculture (3).

The search for antiquorum sensing compounds is possible by using various bioassays (10). Some of the most common bioassays are those performed with *Chromobacterium violaceum* CV026. This bacterium is a mutant of the ATCC 31532 strain, which is deficient in the production of *N*-hexanoyl homoserine lactone (C6-HSL), due to a mutation induced by insertion of a mini-Tn5 in the *cviI* gene, *C. violaceum* acyl-homoserine lactone (AHL) synthase, that controls the synthesis of violacein. Therefore, the quorum sensing mechanisms responsible for the formation of this pigment are activated after the exogenous addition of the autoinducer, AHL. This strain can be used as an indicator organism to detect short-chain molecules with structural similarities to C6-HSL, so that they could compete for the receptor and act as indicators of QS inhibition (10-15). Consequently, this approach has generated many reports plant extracts acting asinhibitors of quorum sensing. One of such extracts is *Allium sativum*, which potentiates the action of the antibiotic tobramycin against *Pseudomonas aeruginosa*, improving the outcome of pulmonary infection (16), Other plants from different families whose extracts exhibit QS-inhibiting properties are *Conocarpus erectus* (Combretaceae), *Quercus virginiana* (Fagaceae), *Bucida buceras* (Combretaceae), *Callistemon viminalis* (Myrtaceae), *Tetrazygia bicolor* (Melastomataceae), and *Chamaecyce hypericifolia* (Euphorbiaceae) (17). The aim of this study was to evaluate the antiquorum sensing activity of essential oils (EOs) of three species of the genus *Piper* growing in Colombia.

MATERIALS AND METHODS

The general methodology used in this study included field collection of plant material, isolation of EOs, and determination of their cell growth, and anti-QS activities as described below.

Bacterial strains and culture conditions.

The bacterial strain used in this study was *Chromobacterium violaceum* CV026, which is unable to produce violacein by itself. However, this biosynthesis can be induced by the presence of AHL, whose *N*-acyl side chains have a length of C4-C8. The C6-homoserine lactone (HHL) was dissolved in dimethylsulfoxide (DMSO), and it was used as autoinducer for violacein pigment production (16).

The bacterial strain, which was kept in Eppendorf tubes at -70ºC, was revived in Luria-Bertani agar (LB) (18) and incubated for 18 hours at 30°C. After this process, culture colonies were transferred to LB broth; cell density was read at a wavelength of 620 nm, and then adjusted to 0.5 in the Mc-Farland scale. This value is equivalent to an absorbance of 0.08-0.1 according to the National Committee for Clinical Laboratory Standards (NCCLS) (18-19).

Plant material and essential oil isolation.

Three plant species of the genus *Piper* were used in the assays (*Piper bogotense*, *Piper brachypodom* and *Piper bredemeyeri*). Plant material was collected from several Colombian states (see table 1) according to information provided by ethnobotanical sources. Samples were properly transported to the laboratory of the Centro Nacional de Investigaciones para la Agroindustrialización de Especies Vegetales Aromáticas Medicinales Tropicales, CENIVAM (National Center for Research on Agro-industrialization of Tropical Medicinal Aromatic Plants), at Universidad Industrial de Santander (Industrial University of Santander), Bucaramanga (Colombia), where the essential oils were extracted. The material was classified systematically by botanist José Luis Fernández, from the Institute of Natural Sciences of Universidad Nacional de Colombia,

UNAL (National University of Colombia). The specimens and their respective vouchers were deposited in the UNAL Herbarium. General information about the species, sampling sites, plant parts used in EO extraction, and voucher number are shown in table 1.

Table 1. Name, origin, type of sample, and vouchers of the plants from which the essential oils tested in this study were extracted.

Species	Common name	Sampling sites	State	Part of the plant used in the extraction	Voucher Number
P. bredemeyeri	Cordoncillo	Puerto Bello	Cesar	Leaves	516939
P. bogotense	Matico	Ipiales	Nariño	Leaves	519590
P. brachypodom	Cordoncillo	Quibdó	Chocó	Whole plant	519974

EOs from different parts of the plant (see table 1) were obtained using hydrodistillation, assisted by microwave radiation. For this hydrodistillation, the extraction equipment was placed next to a domestic microwave oven. A flask was connected to a condenser, and both of them were put into the oven. The flask contained the plant material (100 g) and water (2 L). The water was heated for 30 minutes and the resulting oil was isolated by decantation and dried using anhydrous sodium sulfate.

Preparation of essential oil dilutions.

Essential oils were initially dissolved in DMSO and then added to the culture medium to obtain concentrations of 0.01, 0.1, 1, 10, 50, 100, 200, 300, 400, 500, 750 and 1000 μg/mL. The maximum amount of DMSO used in the assays was 0.5%.

Measurement of cell growth

Cell growth inhibition was assessed following the broth dilution method, according to the recommendations of the NCCLS (19). For this, properly labeled tubes (duplicates) were used to deposit a bacterial culture and different essential oil concentrations (tube A), DMSO (tube B), and one containing neither oil nor DMSO (Tube C). Tube B shows the influence of DMSO on the bacterium, and tube C indicates bacterial behavior without the action of EOs and DMSO. Bacterial growth was identical in tubes B and C, thus suggesting that DMSO did not affect the bacterial culture.

The strain was incubated for a period of 18 hours at 30°C, then adjusted to 0.5 according to the Mc-Farland scale, and 100 μL were placed in tubes containing LB broth and different EO dilutions. These tubes were incubated at 30°C for another 24 hours; then, absorbance was measured in a microplate reader at 620 nm (18). The absorbance data were normalized to vehicle-control, for which a cell growth rate of 100% was assumed. The number of colony-forming units (cfu) of CV026 was estimated on LB agar plates. Cells growing at a rate of 80% in comparison with control cells (cells exposed to 400 μg/mL *P. bredemeyeri* essential oil) were greater than 1.6 x 10^6 cfu/mL.

Measurement of antiquorum sensing activity.

Anti-QS activity measurements were performed as follows: a single colony of the biosensor strain was transferred to LB broth, and then allowed to grow at 30°C for 24 hours, and then the culture was adjusted to 0.5 on the corresponding Mc-Farland scale. 100 μL of this suspension were placed in separate tubes containing LB broth and HHL; LB broth, HHL and DMSO; and LB broth, HHL and different dilutions of essential oil (tubes A, B and C respectively, as shown in table 2). These tubes were incubated at a temperature of 30°C for 24 hours (18). All experiments were performed four times.

Table 2. Composition of the tubes for determining antiquorum sensing activity.

	A	B	C	Final Concentration
LB	895 μL	890 μL	890 μL	-
CV026	100 μL	100 μL	100 μL	1 x 10^8 ufc/ mL
HHL	5 μL	5 μL	5 μL	15 μmol/mL
OIL	-	-	5 μL	(Variable)
DMSO	-	5 μL	-	
Total Volume	1 mL	1 mL	1 mL	

Quantifying violacein production.

After the incubation period, the tubes were vortexed to resuspend cells and biofilms; and 300 μL of this suspension were placed in 1.5 mL Eppendorf tubes. The cells were lysed with 300 μL of 10% sodium dodecyl sulfate, vortexed for 2 minutes, and then incubated at room temperature for 5 minutes. Violacein was extracted quantitatively adding 800 μL of a mixture of butanol/water 1:1, stirred for 5 seconds and then centrifuged at 13000 rpm for 5 minutes. Once centrifuged, the violacein, which was present at the upper layer, was carefully removed and its absorbance measured at 585 nm (11). Violacein concentration was normalized to the concentration obtained for the vehicle-control (100%).

Data analysis.

Results are presented as mean ± standard deviation (x ± SD). A probit analysis was used to calculate the 50% inhibitory concentration (IC_{50}) for both cell growth and quorum sensing. This 50% inhibitory concentration was defined as the concentration of essential oil that leads to a 50% reduction of cell growth compared to vehicle-control, and the concentration of essential oil that leads to a 50% reduction of violacein production compared to the amount produced by *C. violaceum* when fully induced by C6-HSL, respectively. The differences between the means of the responses obtained for each tested concentration were evaluated by analysis of variance (ANOVA), after a logarithmic data transformation. Dunn's test was used to perform comparisons against the control group whenever significant differences were found between means. In all cases, the normal distribution and equality of standard deviations of the means were checked using the Kolmogorov-Smirnov and Bartlett tests, respectively. In the absence of normality, mean comparisons between more than two groups were performed by means of the Kruskal Wallys test. For all cases, the level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Cell growth inhibition

The results obtained in this assay are presented in table 3. The EOs of *P. bredemeyeri*, *P. bogotense* and *P. brachypodom* have a minor effect on the growth of *C. violaceum* CV026. Although there is a clear dose-response relationship, and a low reduction (≈ 10%) of cell growth at concentrations below 100 μg/mL, bacterial growth remained greater than 50% even at concentrations greater than 1000 μg/mL.

Antiquorum sensing activity.

The anti-QS activity of the evaluated EOs is shown in figure 1. Anti-QS activity decreases in the following order: *P. bredemeyeri*> *P. brachypodom*> *P. bogotense*. For *P. bredemeyeri* and *P. brachypodom* IC_{50} was lower or similar to the concentration at which bacterial growth showed no significant differences compared to control cells. However, even for *P. bogotense*, the percentage of cell growth associated with the IC_{50} value is at least of 77%, which indicates that the anti-QS activity for these oils is basically independent of cell growth (7).

Table 3. Cell growth of *C. violaceum* CV026 after exposure to different concentrations of EOs from *Piper* species.

[EO] (μg/mL)	*P. bredemeyeri*	*P. brachypodom*	*P. bogotense*
Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
0.01	99.1 ± 0.2	92.7 ± 1.3	99.0 ± 0.3
0.1	96.6 ± 0.4	91.9 ± 1.5	96.8 ± 0.6
1	93.7 ± 0.5	90.8 ± 1.8	94.0 ± 0.5
10	93.1 ± 0.4	90.3 ± 1.3	93.4 ± 0.6
50	91.7 ± 0.5	89.5 ± 1.0	92.0 ± 0.4
100	88.4 ± 0.4*	88.0 ± 1.5*	89.0 ± 0.4*
200	84.5 ± 0.6*	86.5 ± 0.9*	87.6 ± 0.6*
300	82.4 ± 0.5*	85.9 ± 1.2*	84.6 ± 0.2*
400	80.4 ± 0.5*	83.5 ± 1.5*	83.3 ± 0.5*
500	78.7 ± 0.4*	84.2 ± 0.8*	79.0 ± 0.4*
750	70.7 ± 0.4*	87.0 ± 0.9*	78.1 ± 0.3*
1000	67.0 ± 0.5*	86.3 ± 1.0*	77.0 ± 0.4*
IC_{50}	> 1000	> 1000	> 1000

* Significant differences when compared to control vehicle-treated cells (ANOVA; Dunn's test)

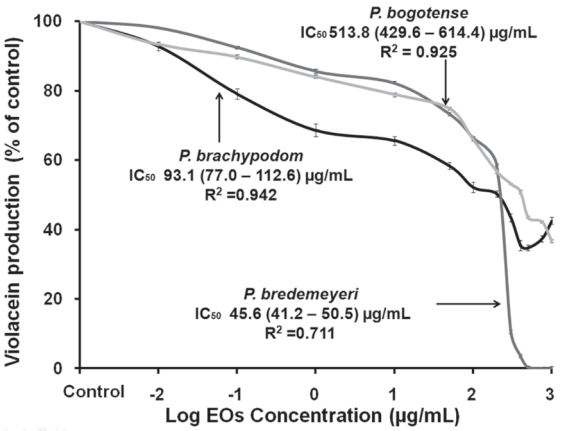

Figure 1. Inhibition of violacein production by EOs isolated from several species of the genus *Piper* using *C. violaceum* CV026 as an indicator strain. IC_{50} values are presented as the mean value (95% confidence interval). R^2, regression coefficient for probit analysis.

The chemical composition of the EOs evaluated in this study has been previously reported in the literature, and some of the major constituents (>10%) present in these species are: Sabinene/β-pinene and α-pinene for *P. bredemeyeri*; trans-β-caryophyllene and caryophyllene oxide for *P. brachypodom*; trans-sabinene hydrate and α-phellandrene for *P. bogotense* (20, 21). This presence clearly reveals the wide variety of chemical constituents that can be found in these species. Interestingly, the most active oil (*P. bredemeyeri*) has a high concentration rate (> 10%) of sabinene/β-pinene/α-pinene molecules, which are absent in *P. brachypodom* and *P. bogotense*, with

the exception of α-pinene, which is present in *P. bogotense* at a concentration rate of 8.7%.

Piper species are traditionally used to treat diseases such as vaginitis and intestinal disorders; although, they have been shown to possess various pharmacological properties such as antiviral, anti-inflammatory, antibacterial, and antifungal activities (22-24). In addition, EOs from this genus inhibit the growth of a large group of microorganisms that cause important human infections such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli;* and the fungi *Trichophyton mentagrophytes*, *Candida albicans*, *Aspergillus flavus* and *Aspergillus fumigatus* (25).

There are several reports in the literature that reveal anti-QS activity in some essential oils, including rosemary, rose, geranium, lavender and clove (26, 27). These oils have constituents that are also present in the EOs evaluated in this study. For instance, α-pinene is present in rosemary oil; whereas linalool and α-humulene are present in rose oil, and limonene in lavender oil (28-30).

Different mechanisms have been proposed to explain the interference of quorum sensing-depending processes by natural products . Some of these mechanisms are the inhibition of signal molecule biosynthesis (1,5) or AHL signal reception (1,5), and the enzymatic inactivation and biodegradation of quorum sensing molecules (31). Although the mechanisms through which EOs inhibit HHL-activated QS systems are not known, the apolar nature and relative size of the components of these oils (similar to HHL) could represent an argument to suggest that these mixtures might be acting through a possible competitive inhibition with the HHL receptor. However, further studies are needed to examine this hypothesis.

The results presented in this paper indicate that the three evaluated EOs are able to inhibit quorum sensing by using the sensor strain *C. violaceum* CV026. This fact suggests that these oils are promising candidates for antibacterial drug development, and could provide an alternative to improve the response to antibiotics, through the intervention of a mechanism such as quorum sensing. Even though, there is still no evidence associated with bacterial resistance (5) for such mechanism.

CONCLUSION

The essential oils of *Piper bredemeyeri*, *Piper bogotense* and *Piper brachypodom* are capable of inhibiting quorum sensing on *C. violaceum* CV026, and these effects occur at concentrations that induce low disruption of cell growth on the sensor strain. Accordingly, these oils are good candidates for the development of anti-QS molecules with potential applications for the control of bacterial diseases mediated by quorum sensing.

ACKNOWLEDGMENTS

The authors thank Colciencias-CENIVAM-Universidad de Cartagena (Project RC-432-2004), Vice-Rectory for research of the Universidad de Cartagena (Research Group Support Program, 2009-2010), Cartagena, Colombia; Dr. Robert McLean, Department of Biology, Texas State University – San Marcos for donating the bacterial strain *C. violaceum* CV026; and Ana Carolina Barreto and Rosa Baldiris Ávila for their invaluable professional support.

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