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Research Paper

Composition, anti-quorum sensing and antimicrobial activity of essential oils from *Lippia alba*

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Abstract

Many Gram-negative pathogens have the ability to produce N-acylhomoserine lactones (AHLs) as signal molecules for quorum sensing (QS). This cell-cell communication system allows them to coordinate gene expression and regulate virulence. Strategies to inhibit QS are promising for the control of infectious diseases or antibiotic resistant bacterial pathogens. The aim of the present study was to evaluate the anti-quorum sensing (anti-QS) and antibacterial potential of five essential oils isolated from *Lippia alba* on the Tn-5 mutant of *Chromobacterium violaceum* CV026, and on the growth of the gram-positive bacteria *S. aureus* ATCC 25923. The anti-QS activity was detected through the inhibition of the QS-controlled violacein pigment production by the sensor bacteria. Results showed that two essential oils from *L. alba*, one containing the greatest geranial:neral and the other the highest limonene:carvone concentrations, were the most effective QS inhibitors. Both oils also had small effects on cell growth. Moreover, the geranial/neral chemotype oil also produced the maximum zone of growth inhibition against *S. aureus* ATCC 25923. These data suggest essential oils from *L. alba* have promising properties as QS modulators, and present antibacterial activity on *S. aureus*.

Key words: violacein, *Chromobacterium violaceum*, limonene, carvone, geranial, neral.

Introduction

The increasing rate of multi-drug-resistant pathogenic bacteria drastically reduced the efficiency of conventional antibiotics. This multi-drug resistance is now recognized as a global health problem (Sipahi, 2008; Köhler *et al.*, 2010; Chong *et al.*, 2011), as many pathogens have the ability to develop antimicrobial resistance through different mechanisms, including random mutations, and the dissemination and interchange of antibiotic resistance genes between diverse pathogenic bacteria (Issac Abraham *et al.*, 2011; Kaufman, 2011). Thus, there is a pressing need for the development of novel therapeutic measures to prevent infection among drug-resistant bacterial pathogens (Musthafa *et al.*, 2010).

A promising approach is to target bacterial cell-to-cell communication, commonly known as quorum sensing (QS). This is a process that bacteria use to sense information from other cells. The "language" utilized for this intercellular communication is mediated by extracellular signaling molecules called autoinducers (AIs), which accumulate in the environment in proportion to cell density (Kabir *et al.*, 2010; Yang *et al.*, 2010; Deep *et al.*, 2011; May *et al.*, 2011; Krishnan *et al.*, 2012). Many physiological processes in the bacteria including luminescence, virulence, motility, sporulation, biofilm formation, development of genetic competence, production of secreted proteolytic enzymes, synthesis of peptide antibiotics and fluorescence are coordinated by QS (Singh *et al.*, 2009; Truchado *et al.*, 2009; Rocha-Estrada *et al.*, 2010).

In general, each bacteria species produces and responds to a unique autoinducer signal. Gram-negative and Gram-positive bacteria use acylated homoserine lactones (AHLs) and oligopeptides as autoinducers, respectively

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(Xavier and Bassler, 2003). Modulation of the physiological processes controlled by AHLs occurs according to cell density and growth phase; this situation induces expression of QS genes (Whitehead *et al.*, 2001). The central components of AHL-driven QS systems are typically members of the LuxI and LuxR protein families. The first one generates homoserine lactones (AHLs), and the second protein family activates or represses the transcription of specific genes, *e.g.* the expression of virulent genes (Xavier and Bassler, 2003; Williams *et al.*, 2007; Morohoshi *et al.*, 2008) in bacterial pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Salmonella typhimurium*, *Yersinia enterocolitica*, among others; helping these cells to succeed during host infection (Khan *et al.*, 2009; Musthafa *et al.*, 2010). The interruption of this communication system can attenuate microbial virulence because many important human pathogens depend on QS signaling systems to coordinate expression of virulence genes (Zhang and Dong, 2004; Vattem *et al.*, 2007; Jaramillo-Colorado *et al.*, 2011; Siddiqui *et al.*, 2012). Strategies designed to interfere with these signaling systems will likely have broad applicability in the biological control of QS-dependent bacterial infections (Truchado *et al.*, 2009; Ditu *et al.*, 2011).

The importance of QS during bacterial pathogenesis has motivated research of inhibition of this mechanism through the use of anti-QS compounds (Choo *et al.*, 2006). These approaches provide the advantage of interfere with this communication system and control the infectious bacteria without halting their growth, thus avoiding the development of bacterial resistance to antibiotics (Debler *et al.*, 2007; Truchado *et al.*, 2009; Christiaen *et al.*, 2011). The process of inactivation or degradation of QS signal molecules (AHL) is called QS inhibition or quorum quenching (QQ). QQ can be achieved in several ways such as through the enzymatic destruction of QS signal molecules, the development of antibodies to QS signal molecules or via agents which block QS (Chan *et al.*, 2011).

Halogenated furanones produced by the benthic marine macroalga *Delisea pulchra* were the first identified anti-QS compounds, for their role in inhibiting biofilm (Teplitski *et al.*, 2000; Choo *et al.*, 2006). Subsequently, other plant-derived anti-QS compounds such as oroidin, ursolic acid, naringenin, cinnamaldehyde, salicylic acid, methyl eugenol, and extracts from garlic and edible fruits, have shown antibiofilm properties against several pathogens (Issac Abraham *et al.*, 2011). In this group, essential oils derived from medicinal and food plants have attracted widespread interest in the search of microbial control alternatives. However, these oils, a rich source of diverse bioactive compounds, have been little studied to ascertain their anti-QS activity (Khan *et al.*, 2009). In this context, the present study was carried out to evaluate the anti-QS activity and cytotoxicity of essential oils from *Lippia alba*, using the bacteria *Chromobacterium violaceum* CV026.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study, *Chromobacterium violaceum* CV026 and *Chromobacterium violaceum* ATCC 31532 (McClean *et al.*, 1997), were kindly provided by Professor Robert J.C. McLean, Texas State University, USA. N-Acyl-homoserine lactone (C6-HSL) was purchased from Sigma (Buchs, Switzerland), aliquoted and used at 15 μM in *C. violaceum* CV026 cultures to induce violacein production. The bacterium was cultivated aerobically and maintained in Luria Bertani (LB) medium at 30 °C; the turbidity was measured at 620 nm and adjusted to match a 0.5 Mc-Farland density standard, which produces approximately 1 x 10^8 cfu/mL according to the Clinical and Laboratory Standards Institute (CLSI) (Choo *et al.*, 2006; CLSI, 2009).

Bacterial strains were reactivated and plated onto LB agar, and then incubated during 18 to 24 h at 30 °C. Colonies were checked by macroscopic (morphology, color and consistency) and microscopic characteristics (Gram staining), and also phenotipically identified. BBL CRYSTAL Enteric/Nonfermenter (E/NF) Identification (ID) kit, from Becton & Dickinson, was used to identify nonfermenting Gram-negative bacteria (Holmes *et al.*, 1994). Strains were stored at -70 °C with 80% glycerol and subcultured twice on Sabouraud agar (Difco) prior to testing anti-QS to confirm its purity and viability.

Plant material and essential oils isolation

Plant specimens were collected from several locations of Colombia. Samples were stored in the dark, transported to the laboratory, washed and used fresh with minimal light exposure. Plant material was identified by an expert botanist from the Institute of Natural Sciences at the National University of Colombia (Bogotá). A voucher of each species was deposited at the Institute's Herbarium. Essential oils were extracted by steam distillation (SD) or microwave assisted hydrodistillation (MWHD) and characterized by GC-MS using a previously reported method (Stashenko *et al.*, 2004). Sampling sites and extraction methods for essential oils are shown in Table 1.

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, 10, 100, 200, 300 μg/mL. The maximum concentration of DMSO used in the assays was 0.5% (Olivero *et al.*, 2011).

Measurement of cell growth

Broth dilution method was used to determine cell growth inhibition (CLSI, 2009). Briefly, a bacterial strain CV026 inoculum was exposed to different dilutions of essential oils or pure DMSO during 18 h at 30 °C (Choo *et al.*,

Table 1 - Essential oils from *Lippia alba* used in this study.

Code[a]	Sampling site	Sample type	Extraction type	Voucher Number
VEsrW01E	Sacabeña, Arauca	Whole plant	MWHD	512084
VEmcT02E	Anolaima, Cundinamarca	Whole plant	SD	484650
VEboW02E	Colorado, Bolívar	Whole plants	MWHD	512272
VEbgW03H	Bucaramanga, Santander	Leaves	MWHD	512077
VEbgW01E	Bucaramanga, Santander	Whole plant	MWHD	480750

Notes: [a] Code assigned by CENIVAM to *Lippia alba* essential oils isolated in different locations or extracted using a specific method.
SD: steam distillation; MWHD: microwave assisted hydrodistillation.

2006) to test their effect on cell growth. After the incubation period, the cell density was read at 620 nm and the absorbance data were normalized to vehicle-control, for which it was assumed 100% of cell growth. *C. violaceum* ATCC 31532 was used as control to ensure reproducible results.

Anti-quorum sensing activity

The assays were done as described in the methodology proposed by Choo *et al.* (2006) and McLean *et al.* (2004). Production of violacein by the mutant CV026 was possible upon external addition of C6-HSL. To investigate the inhibitory effects of *L. alba* essential oils on this process, cells were treated simultaneously with diverse concentrations of the oils and the violacein production was measured.

An overnight culture of *C. violaceum* CV026 (in LB broth, 30 °C) was adjusted to an OD_{620} of 0.1, and then 100 μL of bacterial suspension was added to sterile Eppendorf tubes containing 890 μL of LB media supplemented with 5 μL of C6-HSL (15 μM), and 5 μL of essential oil to achieve final concentrations of 0.01, 0.1, 10, 100, 200 and 300 μg/mL. Control tubes received 5 μL of 0.5% DMSO. Tubes were covered with aluminum foil and incubated aerobically for 24 h. The experiments were performed by triplicates.

Quantification of violacein production

The extent of violacein production by *C. violaceum* CV026 in the presence of control (DMSO) or essential oils from *Lippia alba* was carried out in the presence of C6-HSL at a working concentration of 15 M. First, 300 μL of cultures were placed in Eppendorf tubes and lysed by adding 300 μL of 10% SDS, mixing for 5 seconds with vortex, and incubating at room temperature during 5 min (Blosser and Gray, 2000; Khan *et al.*, 2009). This solution was then mixed with 800 μL of 1-butanol/water (1:1), vortexed for 5 seconds and centrifuged at 13 000 rpm for 5 min. The precipitate was discarded, the upper (butanol) phase containing the violacein was collected, and the absorbance was read at 585 nm (McLean *et al.*, 2004; Choo *et al.*, 2006). The violacein concentration generated in the absence of essential oil was used as positive control and all other measurements were normalized to this value. Each experiment was performed in triplicate.

Antibacterial activity assay

The antibacterial potential of essential oils from *Lippia alba* against *S. aureus* ATCC 25923 was performed through disk diffusion method in Mueller-Hinton agar (MHA; Difco) by following the method specified by the Clinical and Laboratory Standards Institute (CLSI, 2009). The bacterial strain *S. aureus* ATCC 25923 was cultivated in LB for reactivation. Wells (diameter of 6 mm) were made on solidified agar plates. Each pure essential oil (5 and 10 μL) was loaded into the wells, and then the bacteria was inoculated over the whole surface of the agar plate (Krishnan *et al.*, 2012). The plates were incubated during 18 h at 4 °C for the diffusion of the essential oils on the culture medium, and subsequently incubated under aerobic conditions at 37 °C for additional 24 h, followed by measuring the zone of growth inhibition (Issac Abraham *et al.*, 2011).

Gas chromatography-mass spectrometry (GC-MS) analysis

The composition of the essential oil was obtained as previously described (Jaramillo-Colorado *et al.*, 2011) with minor modifications. In short, 20 μL of the oils were dissolved in $CHCl_2$ to 1 mL. One μL of the solution was injected into an Agilent Technologies 6890 Plus (Palo Alto, CA) GC coupled to an Agilent Technologies MSD 5975 selective detector mass equipped with a split/splitless injector port (1:50 split ratio), an injector Agilent 7863, and a data system HP ChemStation. The column had the following characteristics: 30 m, 0.25 mm i.d., and 0.25 μm stationary phase with 5% phenyl poly(methylsiloxane). The oven temperature was set at 45 °C for 5 min, then increased 4 °C/min up to 150 °C during 2 min, then to 5 °C/min up to 250 °C for 5 min, and finally at 10 °C/min up to 275 °C. Helium was used as a carrier gas at 1 mL/min. Identification of compounds was based on Kováts indices (Ik) and by comparison of the mass spectra with those present in available databases.

Data analysis

Results are presented as mean ± standard deviation. Probit analysis was used to calculate the half maximal inhibitory concentration (IC_{50}), for both cell growth and QS, defined as the concentration of essential oil that leads to a 50% reduction of cell growth as compared to vehicle-control, and the concentration of essential oil that leads to a 50% reduction of violacein production as compared to the amount produced by *C. violaceum* when fully induced by C6-HSL, respectively (Olivero *et al.*, 2011). The differences between the means of the responses obtained for each tested concentration were evaluated by analysis of variance (ANOVA), after logarithmic data transformation. Comparisons against the control group were performed if significant differences between means there were found, using Dunn's test. 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 Kruskal Wallys test. For all cases, the level of significance was set at $p < 0.01$.

Results

Cell growth inhibition

The antimicrobial activity of the *Lippia alba* essential oils was analyzed using *C. violaceum* CV026 as indicator microorganism, in order to evaluate whether the inhibition of violacein production owed to the microbial growth reduction or AHL inhibition. The results of the cell growth inhibition of CV026 induced by essential oils from *L. alba* are shown in Figure 1. In all cases, there is a clear concentration-response relationship. At 100 μg/mL, the growth of CV026 varied between 53.4 and 82.6% compared to vehicle control. Greater oil concentrations produced proportional impact in cell growth, except for the essential oil containing the highest geraniol content (9.5%) (VebgW03H), which allowed 23.5% cell growth at 200 μg/mL, and only 15.4 ± 0.8% at 300 mg/mL (Figure 1D).

Quorum sensing inhibition

The inhibitory activity of essential oils from *Lippia alba* against bacterial QS was evaluated by testing the violacein production by *C. violaceum* CV026 (Figure 1). Pigment production data normalized against cell growth (OD_{620}) are depicted in Figure 2. Two out of five tested essential oils presented promising QS-inhibition capabilities. The greatest QS inhibition was observed for the *L. alba* with the highest geranial and neral concentration (VEboW02E) (Figure 1C), followed by that with the greatest limonene and carvone contents (VEmcT02E) (Figure 1B), with IC_{50} values of 0.62 (0.53-0.72) and 2.24 (1.98-2.54) μg/mL. These oils were followed in activity by VEsrW01E (Figure 1A) and VEbgW01E (Figure 1E), which had similar QS inhibitory action, but greater than that observed for VEbgW03H, for which the QS data almost matched that for cell growth (Figure 1D). These results revealed the ability of essential oils from *Lippia alba* to inhibit QS. Moreover, as presented before, the anti-QS activity for these essential oils is independent from their effect on cell growth.

Antibacterial activity assay

To validate some aspects of the traditional uses of the tested bioactive products as antimicrobial agents, essential oils were tested against the bacterial human pathogen *S. aureus*. Regarding the disk diffusion assays, most of the essential oils showed broad zones of growth inhibition against *S. aureus* ATCC 25923 at 10 and 5 μL of each pure essential oil (Figure 3). VEboW02E produced the maximum zone of growth inhibition (46 ± 0.8 mm) (Figure 3D), followed by VEbgW03H with 32 ± 1.0 mm, and VEsrW01E with 31 ± 1.7 mm (Figures 3A and 3E). These findings show that essential oils from *L. alba* do possess antibacterial activity at tested concentrations against Gram-positive bacteria such as *S. aureus* ATCC 25923.

Lippia alba oil composition

The GC-MS analysis of evaluated *L. alba* samples are presented in Table 2. The main constituents were identified as limonene, neral, carvone, geraniol, bicyclosesquiterlandrene, geranial, piperitenone, β-bourbonene, and trans-β-caryophyllene.

Discussion

In many Gram-negative bacteria, a number of bacterial traits, including virulence and pathogenicity, are regulated by acyl homoserine lactone (AHL)-mediated QS system (Khan *et al.*, 2009). The quenching of bacterial cell-cell communication could be a promising strategy to attenuate the expression of virulence genes, and thus for the control of pathogenic infections (Issac Abraham *et al.*, 2011). Although several medicinal properties of *Lippia alba* such as antiviral, antibacterial, antifungal and antiparasitic have been investigated so far (Stashenko *et al.*, 2004; Hennebelle *et al.*, 2008; Ara *et al.*, 2009; López *et al.*, 2011), its ability to prevent QS has been partially studied.

The results of this study revealed that, in the absence of significant effects on cell growth, essential oils from *Lippia alba* have the capacity to significantly inhibit a QS system based on the reduction of violacein production in CV026. Recently we have reported that two of the oils studied here (samples VEsrW01E, voucher 512084, and VEboW02E, voucher 512272) inhibited QS in a model based on sensor strains *P. putida* (pKR-C12) and *E. coli* (pJBA132) (Jaramillo-Colorado *et al.*, 2011).

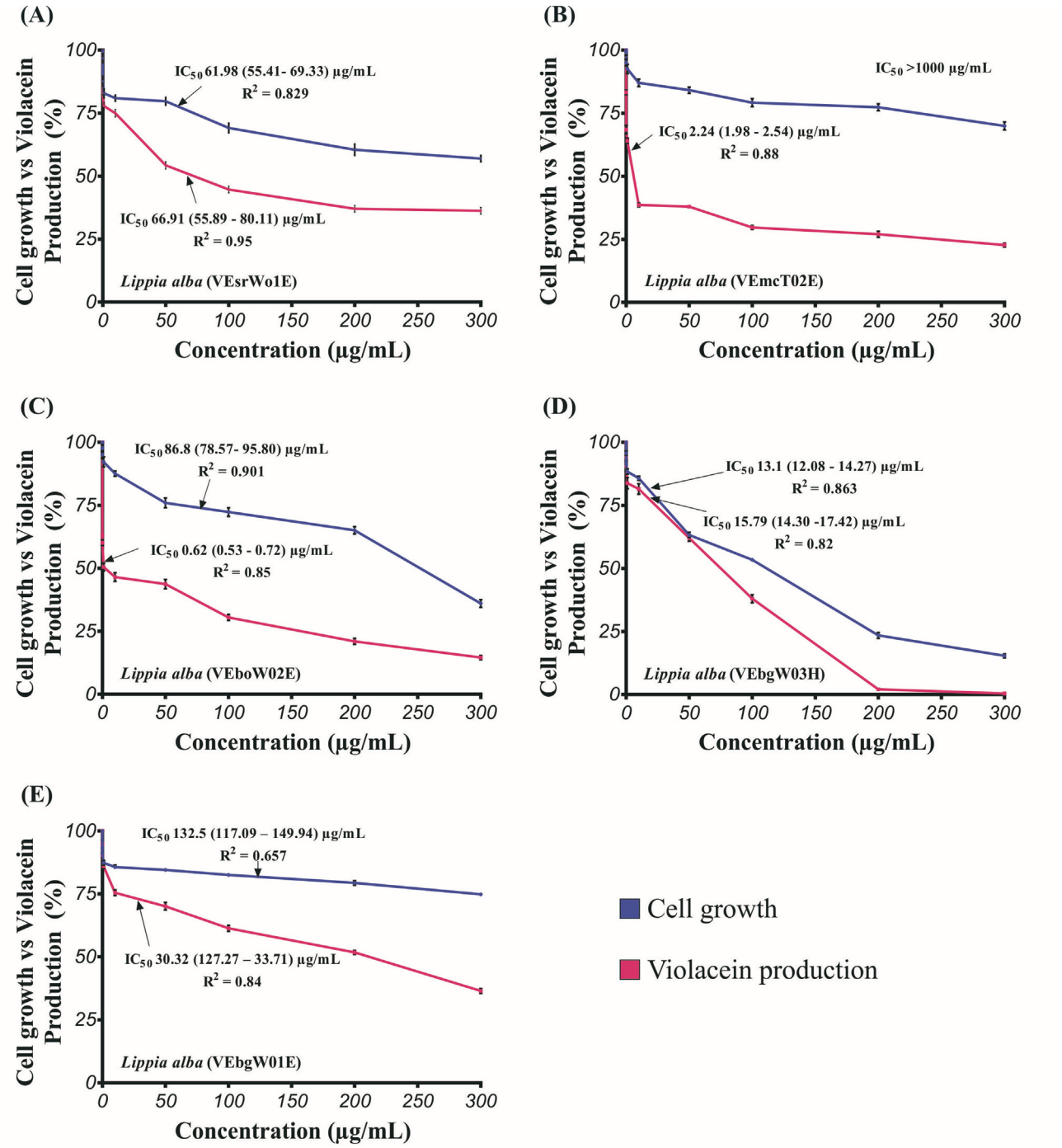

Figure 1 - Effect of essential oils from *Lippia alba* on cell growth and violacein production in *C. violaceum* CV026 exposed to essential oils. (A) VEsrWo1E; (B) VEmcTo2E; (C) VEboW02E; (D) VEbgW03H and (E) VEbgW01E. IC_{50} values are presented as the mean value (95% confidence interval). R^2 (regression coefficient) were done for probit analysis. Vertical bars represent means of three replicates ± standard deviation.

These anti-QS properties have also been found for natural plant extracts such as that of *Lotus corniculatus* (Choo *et al.*, 2006). Similarly, anti-QS compounds have also been reported for *Pseudomonas aeruginosa*, including molecules isolated from *Pisum sativum*, *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras* and *Acalypha alopecuroidea* (Musthafa *et al.*, 2010). Szabó *et al.* (2010) evaluated the effect of several essential oils on bacterial growth and QS, using the sensor strain *C. violaceum* CV026, showing that rose, geranium, lavender and rosemary oils were the most potent QS inhibitors. Eucalyptus and citrus oils moderately reduced

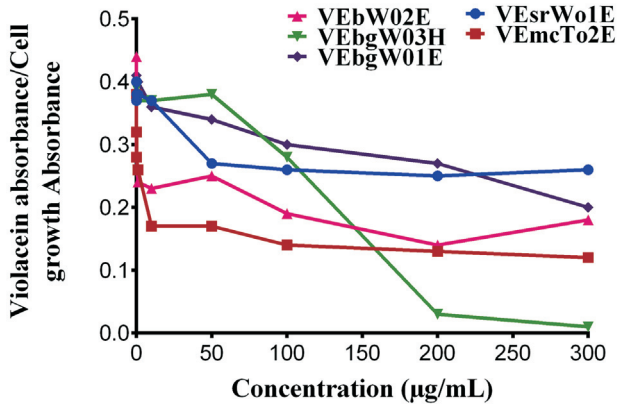

Figure 2 - Violacein production normalized against bacterial concentration (OD_{620}) in *C. violaceum* exposed to essential oils from *Lippia alba*.

pigment production by CV026, whereas the chamomile, orange and juniper oils were ineffective. Choo *et al.* (2006) conducted studies about inhibition of QS signals with vanilla extract, being violacein production reduced by up to 87.73 and 98.41% in 1 and 2% extract, respectively; furthermore, the main chemical components of these extracts had structural similarity to the natural autoinducers or furanone derivatives.

The composition of the essential oil varies greatly, suggesting the existence of a high number of chemotypes. According to Hennebelle *et al.* (2008), there are at least seven chemotypes on the basis of composition and possible common biosynthetic pathways between different oils. Chemotype I had citral, linalool and β-caryophyllene, as the main constituents; chemotype II, tagetenone; the most

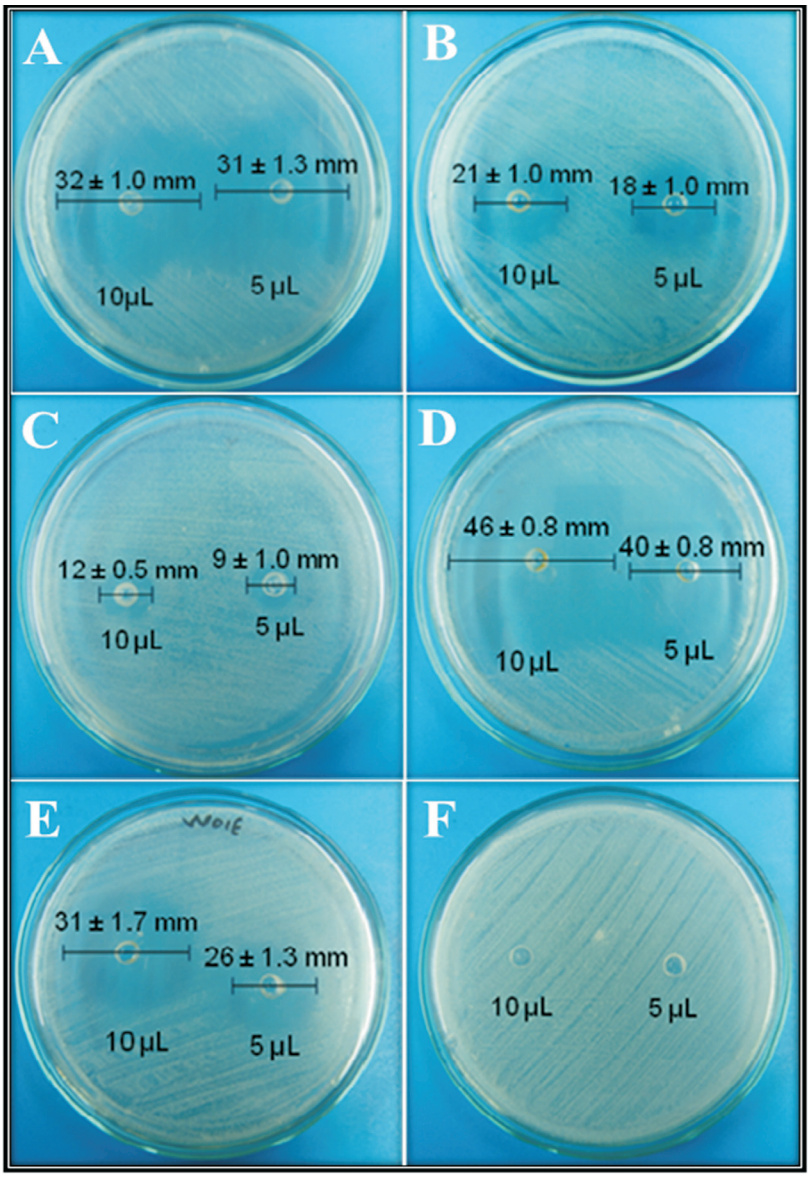

Figure 3 - Antibacterial activity of essential oils from *Lippia alba* on *S. aureus* ATCC 25923 using the agar diffusion test. (A) VEbgW03H; (B) VEbgW01H; (C) VEmcT02E; (D) VEb0W02E; (E) VEsrW01E and (F) DMSO.

Table 2 - Major components of essential oils from *Lippia alba* as identified by Gas chromatography- mass spectrometry.

Components	I_k DB-5	Essential oils from *Lippia alba* (%)				
		VEsrW01E	VEmcT02E	VEboW02E*	VEbgW03H	VEbgW01E*
6-Methyl-5-hepten-2-one	986	1.9	-	4.1	2.6	-
Limonene	1034-1041	22.4	37.0	3.2	3.0	25.8
Linalool	1100-1102	0.6	0.7	2.0	1.4	0.9
Neral	1239-1248	10.4	-	21.5	19.5	-
Geraniol	1249-1258	-	-	3.9	9.5	-
Carvone	1251-1258	25.3	27.9	-	-	49.6
Piperitone	1260-1264	1.1	0.1	-	-	3.2
Geranial	1271-1277	10.4	-	28.9	23.3	-
Piperitenone	1343-1349	2.2	1.6	-	-	6.1
Geranyl acetate	1373-1379	0.4	0	1.6	3.6	0
β-Elemene	1385-1398	1.7	0.1	0.9	2.2	0
β-Bourbonene	1391	-	5.3	-	-	2.4
trans-β-Caryophyllene	1430-1436	2.4	1.4	7.3	6.6	0.1
β-Guaiene	1442-1447	1.3	-	1.8	1.1	-
trans-β-Farnesene	1451-1456	0.2	1.8	0.1	0.4	0.7
Bicyclosesquifelandrene	1490-1496	8.0	12.3	1.9	3.0	1.2
Caryophyllene oxide	1596-1599	0.4	-	2.3	1.3	-

I_k DB-5: Kovats retention index for DB-5.
*: The composition of these essential oils has been previously reported (Jaramillo-Colorado *et al.*, 2011).

common case for chemotype III were limonene and carvone or related monoterpenic ketones; chemotype IV, myrcene; chemotype V, γ-terpinene; chemotype VI camphor-1, 8-cineole and chemotype VII, estragole. Therefore, the essential oils of *L. alba* studied here were classified as citral (geranial-neral) and carvone/limonene chemotypes, thus corresponding to chemotypes I and III, respectively.

L. alba species is characterized by the variability in the chemical composition of its essential oils, which vary, as it has been presented here, according to the part of the plant employed in the distillation, the state of development, the geographic location, the characteristics of the soil, climate, and other local conditions (Stashenko *et al.*, 2004). Most of the essential oils have one or a few major constituents and a variety of other minor components. Thus, mode of action of these mixtures on the QS system is uncertain (Khan *et al.*, 2009), although it may be speculated that it probably involves a synergistic action of the majority and minority compounds identified in the essential oils.

To date, known mechanisms of QS inhibition include: competitive binding of signal-like molecules to cognate receptors, as in the case of furanones; enzymatic signal degradation, as seem with acyl homoserine lactone (AHL) acylases, and inhibition of reception signal molecules (Sio *et al.*, 2006; Taganna *et al.*, 2011). Our results have shown that essential oils are a diverse and suitable source of QS inhibitor compounds. The presence of molecules with different structures in the oils, allows a wider spectrum of bioactivity. Several studies on the essential oils from *L. alba* bioactivities have revealed their antiviral, antibacterial, antifungal and antiparasitic activities, thus sustaining their use in the treatment of diseases of microbial origin (Escobar *et al.*, 2010; López *et al.*, 2011). A recent report by our group has revealed the anti-QS activity of essential oils isolated from *L. alba* and other species. In this case, although an approach with different sensor plasmids (Jaramillo-Colorado *et al.*, 2011) was used, similar findings were obtained, reinforcing the idea that examined essential oils do have promising action as anti-QS inhibitors.

Antiseptic activity has been a promising issue for *Lippia alba*. Several studies have reported the antimicrobial and antifungal activities of this essential oil extracted from two chemotypes. The carvone chemotype has been reported to be active against several microorganisms, particularly against Gram-positives strains from human clinical isolates. On the other hand, the linalool chemotype has been successfully proven against *Candida albicans* (Hennebelle *et al.*, 2008; Jaramillo-Colorado *et al.*, 2011).

Due to the high antimicrobial activity of monoterpenes and sesquiterpenes from essential oils (Oliveira *et al.*, 2006), it can be suggested that the antimicrobial activity of VEboW02E and VEbgW03H is probably related to the high content of oxygen-containing monoterpenes, represented mainly by aldehydes and alcohols, such as neral/geranial (Oliveira *et al.*, 2006).

In short, these results suggest the essential oils from *Lippia alba* have interesting anti-QS and antiseptic activities. Therefore, new researches must be undertaken in order to elucidate the possible mechanisms involved.

Conclusion

Essential oils from Colombian flora, specifically *Lippia alba* have noticeable anti-QS and antimicrobial activities, which may make them targets for the development of drugs to fight bacterial infections.

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