Natural Product Research: Formerly Natural Product Letters

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gnpl20

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Published online: 21 Sep 2011.

To cite this article: Beatriz Jaramillo-Colorado , Jesus Olivero-Verbel , Elena E. Stashenko , Irene Wagner-Döbler & Brigitte Kunze (2012) Anti-quorum sensing activity of essential oils from Colombian plants, Natural Product Research: Formerly Natural Product Letters, 26:12, 1075-1086, DOI: 10.1080/14786419.2011.557376

To link to this article: http://dx.doi.org/10.1080/14786419.2011.557376

Natural Product Research
Vol. 26, No. 12, June 2012, 1075–1086

Anti-quorum sensing activity of essential oils from Colombian plants

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(*Received 26 June 2010; final version received 20 January 2011*)

Essential oils from Colombian plants were characterised by GC–MS, and assayed for anti-quorum sensing activity in bacteria sensor strains. Two major chemotypes were found for *Lippia alba*, the limonene-carvone and the citral (geranial-neral). For other species, the main components included α-pinene (*Ocotea* sp.), β-pinene (*Swinglea glutinosa*), cineol (*Elettaria cardamomun*), α-zingiberene (*Zingiber officinale*) and pulegone (*Minthostachys mollis*). Several essential oils presented promising inhibitory properties for the short chain AHL quorum sensing (QS) system, in *Escherichia coli* containing the biosensor plasmid pJBA132, in particular *Lippia alba*. Moderate activity as anti-QS using the same plasmid, were also found for selected constituents of essential oils studied here, such as citral, carvone and α-pinene, although solely at the highest tested concentration ($250\,\mu g\,mL^{-1}$). Only citral presented some activity for the long chain AHL QS system, in *Pseudomonas putida* containing the plasmid pRK-C12. In short, essential oils from Colombian flora have promising properties as QS modulators.

Keywords: essential oil; Colombian plants; quorum sensing

1. Introduction

Essential oils are complex mixtures of volatile compounds isolated from plant materials. They are secondary metabolites chemically derived from terpenes and their oxygenated compounds, and each component contributes to their biological activities. These complex mixtures have different pharmacological properties (Firuzi, Asadollahi, Gholami, & Javidnia, 2010), including anti-inflammatory (Tavares et al., 2010), anti-tubercular (Bueno, Martínez, Stashenko, & Ribon, 2009), antibacterial (Okoh, Sadimenko, & Afolayan, 2010), antifungal (Hashem, Moharam, Zaied, & Saleh, 2010), protective of the gastrointestinal tract (Wang et al., 2008),

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ISSN 1478–6419 print/ISSN 1478–6427 online

http://dx.doi.org/10.1080/14786419.2011.557376
http://www.tandfonline.com

anticonvulsant (Quintans-Júnior, Souza, Leite, Lessa, & Bonjardim, 2008; Viana, do Vale, Silva, & Matos, 2000) and antioxidant properties (Loizzo et al., 2009; Singh et al., 2010).

The antipathogenic properties of plants have received much less attention, but may be just as important in combating disease as their antibacterial counterparts (Adonizio, Downum, Bennett, & Mathee, 2006). The interruption of quorum sensing (QS), or bacterial cell-to-cell communication, is one example of an antipathogenic effect. Since a large number of systems affecting pathogenicity are controlled by QS, interrupting this communication system can render pathogenic bacteria non-virulent (Zhang & Dong, 2004). Inappropriate use or overuse of antibiotics has lead to an increase in antibiotic resistance that has a great impact on human health around the world. The need for new antibiotics with mechanisms of action different from traditional ones is fundamental to increase survival probabilities in clinical infections where resistance is global. One of the existing alternatives to finding new antibacterial compounds relies on a sophisticated bacterial communication process called 'QS' (Raina et al., 2009). This process is important for bacteria to regulate community-wide behaviours including biofilm formation, virulence, conjugation, sporulation and swarming motility (Jayaraman & Wood, 2008).

QS allows bacteria to monitor its cell density through the release of specific signalling molecules called autoinducers. During growth, these chemicals are accumulated in the local environment until reaching a threshold concentration that initiates the signalling required for changes in gene expression, many of them involved in the regulation of a battery of virulence genes (Lynch & Wiener-Kronish, 2008). Gram-negative bacteria use N-acyl homoserine lactones (AHLs) as autoinducers, whereas Gram-positive bacteria rely on peptides, which after binding to specific receptors, initiate the signal cascade leading to the activation of systems that help these cells to succeed during host infection (Li et al., 2007). Based on this novel mechanism of action, QS has been suggested as an opportunity to fight bacterial infection/virulence by means other than growth inhibition, overcoming the problem linked to antibiotic resistance (Hentzer & Givskov, 2003; Lynch & Wiener-Kronish, 2008). Currently, many compounds, both synthetic and natural have been reported to have an anti-QS activity. The first group includes methylthioadenosine analogs (Gutierrez et al. 2009), whereas furanones (Lönn-Stensrud, Landin, Benneche, Petersen, & Scheie, 2009), cinnamaldehyde and substituted cinnamaldehyde (Brackman et al., 2008), are among the natural ones.

Despite the fact that Colombia has an immense biodiversity, few studies have been reported on the biological properties of essential oils from plants grown in this country. Among those, species such as *Lippia alba*, *Ocotea* sp, *Elettaria cardamomum*, *Swinglea glutinosa*, *Myntotachys mollis* and *Zingiber officinale* have been found to possess several biological properties, such as antioxidant, antifungal, antimicrobial, insect repellent, antiprotozoal, etc. (Bueno et al., 2009; Jaramillo, Stashenko, & Martínez, 2010; Olivero, Gonzalez, Güette, Jaramillo, & Stashenko, 2010; Stashenko, Jaramillo, & Martínez, 2004a; Weniger et al., 2001). In this work, we investigated if essential oils from plants grown in Colombia might have a potential as anti-QS substances, and therefore tested their ability to inhibit AHL-based QS.

Table 1. Essential oils from Colombian plants used in this study.

Species	Family	Sampling site	Extraction method	Voucher
L. alba (1)[a]	*Verbenaceae*	Bolívar	MWHD	512272
S. glutinosa	*Rutaceae*	Arauca	MWHD	521530
L. alba (2)	*Verbenaceae*	Cundinamarca	HD	484350
L. alba (3)	*Verbenaceae*	Tolima	MWHD	484350
L. alba (4)	*Verbenaceae*	Tolima	HD	484350
M. mollis	*Labiatae*	Cundinamarca	HD	521089
L. alba (5)	*Verbenaceae*	Arauca	MWHD	512084
Ocotea sp	*Lauraceae*	Boyacá	MWHD	CENIVAM-457[b]
E. cardamomum	*Zingiberaceae*	Antioquia	HD	CENIVAM-450[b]
Z. officinale	*Zingiberaceae*	Caldas	HD	CENIVAM 453[b]

Notes: [a] Numbers in parenthesis refer to *L. alba* essential oil sampled from a different location or extracted using a specific method. [b]Samples with vouchers named CENIVAM are stored in our herbarium located in Bucaramanga (Colombia).
HD: hydrodistillation; MWHD: microwave assisted hydrodistillation.

2. Results and discussion

2.1. *Essential oil composition*

Table 1 shows the essential oils from Colombian plants used in this study. It presents scientific names of the plants, family, geographic site of collection, extraction method and identification number of the species. The major chemical components of the essential oils tested in this study are shown in Figure 1. In the case of *L. alba*, two different chemotypes were found, the limonene-carvone and the citral (geranial-neral). α-pinene and β-pinene were found to be the most abundant components in *Ocotea* sp. and *S. glutinosa*, respectively; whereas cineol, α-zingiberene and pulegone for *E. cardamomun*, *Z. officinale* and *M. mollis*, respectively. Large variations have been observed in the volatile composition of essential oils, depending on the part of the plant employed in the distillation, plant's state of development, geographic location, characteristics of the soil, climate and other local conditions (Stashenko et al., 2004a).

It is clear that the composition of the essential oil of *L. alba* depends on the collection site and the corresponding chemotype. The main constituents of *L. alba* (collected in Cundinamarca, Tolima and Arauca departments) were carvone and limonene. However, in *L. alba* (Collected in Bolivar Department), the main components were geranial and neral (Figure 1). Although quantitative differences are verified, the composition of the essential oils of *L. alba* shows agreement with the existence of different chemotypes as previously described (Bounatirou et al., 2007; Oliveira et al., 2006; Zoghbi, Andrade, Santos, Silva, & Maia. 1998). The principal components of *Ocotea* sp. were α-pinene, p-cymene and β-pinene. This composition is different from that found for *Ocotea quixos* (Ishpingo) from Ecuador, oil that has *trans*-cinnamaldehyde and methyl cinnamate as major constituents (Bruni et al., 2004). More than 350 species belong to the *Ocotea* genus, mainly distributed in the Americas and in Southern Africa, and those are widely known as commercial sources of spicy essential oils.

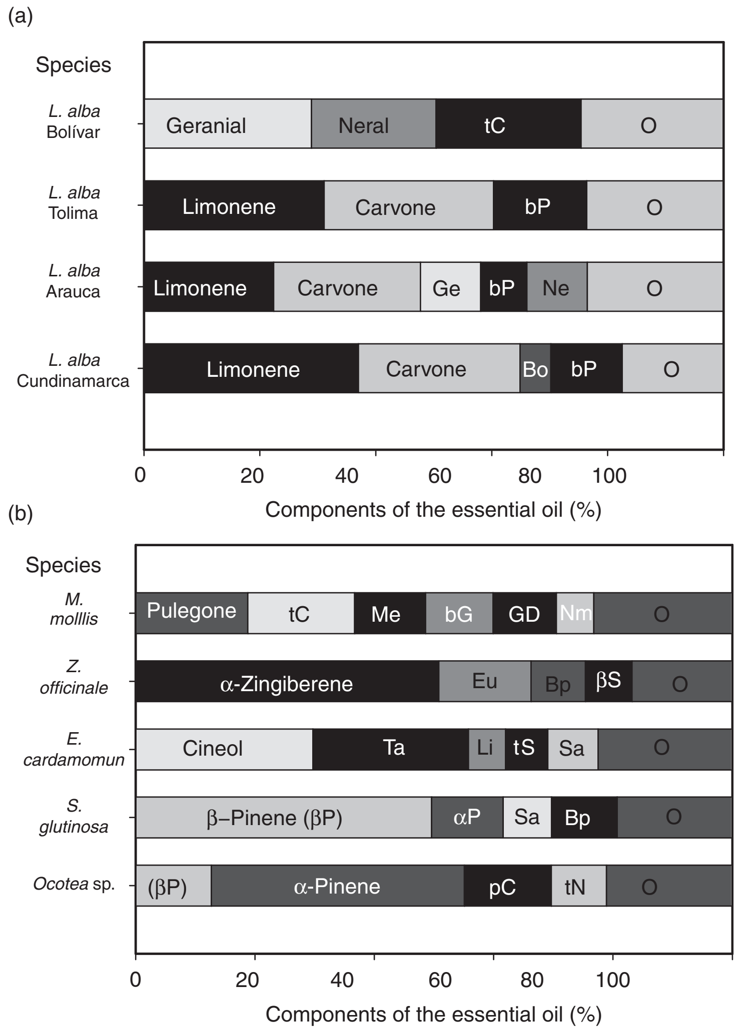

Figure 1. Composition of essential oils from different plants collected in Colombia. (a) Genus *Lippia*. (b) Other genus. Abbreviations. αP, α-pinene; βP, β-pinene; βS, β-sesquiphellandrene; *p*C, *p*-cymene; tC, *trans*-caryophillene; tN, *trans*-nerolidol; bG, bicyclogermacrene; bP, bicyclosesquiphellandrene; Bo, bourbone; Ci, 1,8-cineol; Eu, eugenol; Ge, geraniol; GD, germacrene D; Me, menthol; Nm, neomenthol; Sa, sabinene; Li, linalool; Ne, neral; *t*S, *trans*-acetate sabinile hydrate; Ta, α-terpinyl acetate; O, other components.

The main components found in the volatile oil from *E. cardamomum* were 1,8-cineol and α-terpineol acetate. In contrast, Marongiu, Piras, and Porcedda. (2004) found as majority compounds in *E. cardamomun* α-terpinyl acetate, 1,8-cineole, linalyl acetate, limonene and linalool. Regarding the other studied species, major constituents for Colombian *M. mollis* were pulegone, mentone and *trans*-β-caryophyllene. Alkire, Tucker, and Maciarello (1994) reported the major compounds obtained in the essential oil from ecuatorian *M. mollis:* neomenthol, menthone, menthol and piperitone.

Table 2. Anti-QS of essential oils isolated from Colombian plants.

	Anti-QS activity (% inhibition after 24 h exposure[a])	
Species	*P. putida* (pKR-C12)	*E. coli* (pJBA132)
L. alba (1)	65	18
S. glutinosa	Null	28–73
L. alba (2)	Null	15–84
L. alba (3)	1	72–78
L. alba (4)	Null	61–62
M. mollis	Null	56–65
L. alba (5)	9	44–72
Ocotea sp.	25	71–76
E. cardamomum	31	21–22
Z. officinale	Null	34–69

Notes: [a]Tested concentration was 1.2 mg mL^{-1}. For numbers in parenthesis refer to *L. alba* essential oil sampled from a different location or extracted using a specific method (see Table 1).

For Colombian essential oil from *S. glutinosa* the major components found were: β-pinene, α-pinene sabinene and bicyclosesquiphellandrene. Whereas (E)-nerolidol and caryophyllene oxide were the major components found in the essential oil from Cuban *S. glutinosa* (Pino, Marbot, & Fuentes, 2006). And for Colombian *Z. officinale* were: α-zingiberene, eugenol, bicyclosesquiphellandrene. In comparison, the essential oils obtained by hydrodistillation of the leaves and rhizomes of *Z. officinale* from Malaysia, the leaf oil was clearly dominated by β-caryophyllene, while the oil from the rhizomes was predominantly monoterpenoid, with camphene, geranial and geranyl acetate the three most abundant constituents (Sivasothy et al., 2011).

2.2. *Inhibition of QS*

Anti-QS properties for examined essential oils are shown in Table 2. Differences in activity for essential oils of *L. alba* are clearly dependent on the extraction method and the region where collection took place (Stashenko et al., 2004a; Zoghbi et al., 1998). Most tested oils had anti-QS properties for the sensor plasmid pJBA132, but few were active using the long-chain sensor plasmid pRK-C12. Essential oils of *L. alba* and *Ocotea* sp. were the most active.

Inhibition of QS for different selected constituents of essential oils are presented in Figure 2. Citral, at the highest tested concentration (250 μM), was the only essential oil constituent that presented some activity as a QS inhibitor for the long-chain sensor plasmid pKR-C12 (*P. putida*). However, all examined compounds elicited moderate to good inhibition when examined against the sensor plasmid pJBA132 (*E. coli*). This is not surprising considering that pKR-C12 responds to long-chain AHLs (C12), whereas pJBA132 (*E. coli*) sensors short-chain AHLs (C6) (Wagner-Döbler et al., 2005). Although geranyl acetate has a total number of carbon atoms of 12 ($C_{12}H_{20}O_2$), it does not fit the 'long-chain' requirement because it is a

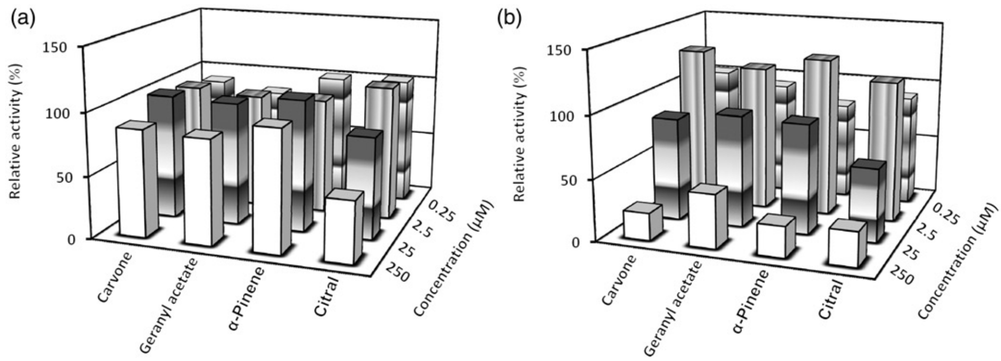

Figure 2. Anti-QS activity of different constituents of essential oils, measured as % activity relative to AHL (100%). QS in (a) *P. putida* (pKR-C12), and (b) *E. coli* (pJBA132).

branched molecule, as the other ones, and this probably prevents it from reaching the appropriate molecular size for binding on the receptor. On the other hand, both geranial and neral (citral) are linear compounds that seem to have some interference with the signalling induced by C_{12}-HSL; however, the mechanisms involved in the observed effects are still unkonwn.

The search for new chemicals that interfere with QS-related mechanisms in bacteria has considerably increased during the last years, opening up an alternative strategy for antimicrobial chemotherapy through the discovery of safer and less antibiotic resistant anti-bacterial drugs (Vattem, Mihalik, Crixell, & McLean, 2007). As a consequence, compounds from plants (Adonizio et al., 2006; Limsuwan, & Voravuthikunchai, 2008), animals (Park, Kaufmann, Bowen, Arbiser, & Janda, 2008), as well as from computational methods such as virtual screening (Kiran, Adikesavan, Cirioni, Giacometti, & Silvestri, 2008), have been found to have good anti-QS properties. Szabó, Varga, Hohmann, Schelz, and Szegedi. (2010) conducted studies about inhibition of QS signals by essential oils, showing that rose, geranium, lavender and rosemary oils were the most potent QS inhibitors. Eucalyptus and citrus oils moderately reduced pigment production by CV026, whereas the chamomile, orange and juniper oils were ineffective. Adonizio et al. (2006) worked with 50 medicinal plants from southern Florida which were screened for anti-QS activity using two biomonitor strains, *Chromobacterium violaceum* and *Agrobacterium tumefaciens*. Of these plants, six showed QS inhibition: *Conocarpus erectus* L. (Combretaceae), *Chamaecyce hypericifolia* (L.) Millsp. (Euphorbiaceae), *Callistemon viminalis* (Sol. ex Gaertn.) G. Don (Myrtaceae), *Bucida burceras* L. (Combretaceae), *Tetrazygia bicolor* (Mill.) Cogn. (Melastomataceae), and *Quercus virginiana* Mill. (Fagaceae).

The results presented here have shown that essential oils are a diverse and suitable source of anti-QS molecules. The presence of molecules with different structures in the oils, also allows a wider spectrum of bioactivity. This is clearly seen with the two *L. alba* chemotypes studied here. The limonene-carvone chemotype has a greater activity against QS mediated by 3-oxo-C_6-HSL and no activity against the system activated by C_{12}-HSL. In contrast, the citral chemotype (geranial-neral), has the opposite preference. Structurally speaking, limonene and carvone are not only cyclic monoterpenes but in terms of total length, they are more similar to a C_6 linear carbon skeleton, and consequently more likely to compete with a C_6-HSL. Moreover, as previously discussed, pKR-C12 has sensitivity for compounds with C_{12}-HSL, although this almost disappears acyl chains shorter than C8 (Steidle et al., 2002), explaining the lack of activity of the *L. alba* limonene/carvone chemotype in the *P. putida* strain.

Although anti-QS properties have not been reported for major components found in assayed essential oils, some of these compounds have been shown to be promising antimicrobial agents. Carvone, for instance, has also been identified as a major constituent in oils with antibacterial properties against both gram positive and gram negative bacteria (Shahat et al., 2008). Moreover, this terpenoid can act synergistically with other antibacterial compounds. Carvone alone, or the essential oil of *Mentha spicata* and *Anethum graveolens*, containing 40.1 and 20.3% carvone, augmented the nitrofurantoin activity against *Enterobacter cloacae* (Rafii & Shahverdi, 2007). As presented here, *Lippia alba* components are recognised as an important source of antimicrobial agents (Hennebelle, Sahpaz, Joseph, & Bailleul, 2008; Oliveira et al., 2006). In fact, not only this species, but in general others belonging to the same genus, have been used in traditional herbal medicine to treat several respiratory problems, including colds and coughs (Viljoen, Subramoney, van Vuuren, Başer, & Demirci, 2005). Despite the fact that biological activity of essential oils is usually linked to the presence of major components (Firuzi et al., 2010), in some cases, those chemicals present in low concentrations could also be responsible for the observed biological activities (Grassmann, Hippeli, Vollmann, & Elstner, 2003). Regarding species different from *Lippia*, other compounds detected in essential oils are also of special interest. It has been reported that pulegone, thymol or cineol-rich essential oils have also been effective against Gram-positive and Gram-negative bacteria (Oumzil et al., 2002; Shapira, & Mimran, 2007).

Despite the good anti-QS properties observed for tested essential oils, reported toxicity, based on the brine shrimp mortality method, suggests that most of the evaluated essential oils were cytotoxic, with LC_{50} values closer or below $20\,\mu g\,mL^{-1}$ (Olivero et al., 2010). Accordingly, additional pharmacological actions for these oils should also be considered. Several investigators have reported the use of *L. alba* as neurosedative (Hennebelle et al., 2008), anticonvulsivant (Viana et al., 2000), antioxidant (Stashenko et al., 2004 a) and antiviral (Andrighetti-Fröhner et al., 2005), among others.

In short, results presented here suggest that essential oils from Colombian flora have interesting anti-QS activities which may make them targets for the development of antimicrobial drugs.

3. Experimental

3.1. *Bacterial strains*

The sensor strains were grown at 37°C on LB medium with $25\,\mu g\,mL^{-1}$ tetracycline (*E. coli* MT102/pJBA132) and $20\,\mu g\,mL^{-1}$ gentamycin (*Pseudomonas putida* F117/pKRC12), respectively.

3.2. *Collection of plant materials and essential oil extraction*

Plant specimens were collected from several locations along Colombia. Sampling sites and extraction method are shown in Table 1. Samples were stored in the dark and transported to the laboratory, washed and used fresh with minimal light exposure. Plant material was identified by botanist Jaime Aguirre Santoro from the Institute of Natural Sciences at the National University of Colombia (Bogotá). A voucher of each species is deposited at the Institute's Herbarium.

3.3. *Extractions*

Essential oils were extracted by hydrodistillation (HD) or microwave assisted hydrodistillation (MWHD). HD was performed in a 5 L-round flask with 500 g of plant material and 4 L of water, using an electric heater (boiling water) for 2 h. The oil, previously saturated with NaCl, was decanted from the condensate and dried with anhydrous sodium sulfate (Stashenko et al., 2004a; Stashenko, Jaramillo, & Martinez, 2004b). MWHD consisted of a hydrodistillation apparatus placed inside a domestic microwave oven (Kendo, 2.45 GHz, 800 W), with a side orifice through which an external glass condenser joined the round flask with the plant material (100 g) and water (2 L), inside the oven. The oven was operated for 30 min at full power, which caused water to boil vigorously and reflux (Paré, Bélanger, & Stafford, 1994; Stashenko et al., 2004a; Stashenko et al., 2004b).

3.4. *GC-MS Analysis*

The essential oil analysis was carried out using an *Agilent Technologies* 6890 *Plus* equipped with a HP-5 MS capillary column ($30\,m \times 0.25\,mm$ i.d. $\times\, 0.25\,\mu m$, d_f), coupled to an *Agilent Technologies* MSD 5973 mass-selective detector. For GC-MS detection, an electron ionisation system with ionisation energy of 70 eV was used. Helium was the carrier gas at a flow rate of $1\,mL\,min^{-1}$. Diluted samples (1:100 v/v, in CH_2Cl_2) of 1.0 µL were injected manually and in the *splitless* mode. Components were identified on the basis of comparison of their relative retention time and mass spectra with those of some standard compounds, NBS75K library data of the GC-MS system, and reported data (Adams, 1995; Davies, 1990).

3.5. *QS inhibition assay*

Inhibition of QS systems driven by AHLs was evaluated using biosensor strains that do not produce AHLs by their own but are capable of sensing their presence through a sensor plasmid which expresses an AHL controlled promoter fused to a gene coding for fluorescence (gfp). *P. putida* F117 (pKR-C12) was used to detect inhibitors of long-chain AHLs (C12), and *E. coli* MT102 (pJBA132) was used for the detection of short chain AHL (C6) inhibitors (Wagner-Döbler et al., 2005). In short, overnight cultures (50 mL) of these strains were diluted with the same volume of accordant fresh medium and incubated for one more hour. These cultures adjusted to an OD_{620} of 1.0 were used for the bioassay. Aliquots of 100 µL were incubated at 30°C in microtiter plates together with 90 µL of the medium, 5 µL of the essential oil (0.025–$1.25\,mg\,mL^{-1}$) or essential oil's component, and 5 µL of the corresponding

AHL (0.25 μg mL^{-1}). Samples containing only the corresponding AHL without test compound were used as positive control and samples containing only solvent (10 μL) with neither test compound nor AHL were used as negative control. After 24 h, fluorescence at an excitation wavelength of 485 nm and a detection wavelength of 535 nm, and growth at an OD_{620} was determined in a Victor 1420 Multilabel Counter. Afterwards the fold induction of fluorescence in the test samples was calculated by dividing their specific fluorescence (gfp_{535}/OD_{620}) by the specific fluorescence of the negative control. Percent of inhibition was calculated by comparing the fold induction of positive control samples (only AHL) to samples with AHL and essential oils. Anti-QS in one experiment was measured in triplicate and data are presented for at least two independent experiments.

Due to the complex and diverse nature of components present in the essential oils, several individual compounds and an isomeric mixture (citral, geranial + neral) that were found both as major components (R-carvone 98%, citral 95%, from Aldrich Chemical Corp., Milwaukee, WI, USA; and α-pinene 98.5% from Fluka,) as well as a minor constituent (geranyl acetate 99%, from Fluka) were also tested for QS inhibition.

4. Conclusions

In short, results presented here suggest that essential oils from Colombian flora have interesting anti-QS activities which may make them targets for the development of antimicrobial drugs.

Acknowledgements

The authors thank Colciencias, Bogota, Colombia (Grant no. RC 432-2004), and the Von Humboldt Alexander Foundation (Germany). The authors also thank Birte Engelhardt for skillfull assistance and Leo Eberl for providing the biosensor strains for the QS tests.

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