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A novel property of propolis (bee glue): Anti-pathogenic activity by inhibition of *N*-acyl-homoserine lactone mediated signaling in bacteria

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ABSTRACT

Ethnopharmacological Relevance: An alternative approach to antibiotics is the development of anti-pathogenic agents to control the bacterial virulome. Such anti-pathogenic agents could target a phenomena known as quorum sensing (QS).
Materials and methods: Six bacterial *N*-acyl-homoserine lactone (AHL)-dependent bioreporter strains were used to evaluate if bee hive glue also known as propolis contains constituents capable of inhibiting QS-controlled AHL signaling. In addition, the effect of propolis on the QS-dependent swarming motility was evaluated with the opportunisitic pathogen, *Pseudomonas aeruginosa*.
Results: Differences in the propolis tincture samples were identified by physiochemical profiles and absorption spectra. Propolis tinctures at 0.0005% (v/v) that do not affect bacteria biosensor growth or the reporter system monitored were exposed to biosensors with and without the addition an AHL. No AHL signal mimics were found to be present in the propolis tinctures. However, when propolis and an inducer AHL signal were together exposed to five *Escherichia coli* and a *Chromobacterium violaceum* biosensor, propolis disrupted the QS bacterial signaling system in liquid- and agar-based bioassays and in C_{18} reverse-phase thin-layer plate assays. Swarming motility in the opportunistic pathogen, *Pseudomonas aeruginosa* PAO1 and its AHL-dependent LasR- and RhlR-based QS behaviors were also inhibited by propolis.
Conclusions: Together, we present evidence that propolis contain compounds that suppress QS responses. In this regard, anti-pathogenic compounds from bee harvested propolis could be identified and isolated and thus will be valuable for the further development of therapeutics to disrupt QS signaling systems which regulate the virulome in many pathogenic bacteria.

1. Introduction

The word propolis, used in Ancient Greece as: *pro* for infront of or at entrance to and *polis* for city or community. It is a chemically complex substance collected by honeybees (*Apis mellifera*) from cracks in bark and leaf buds of regional macroflora (Ghisalberti, 1979; Burdock, 1998). Propolis is a plant resinous substance masticated with bee salivary enzymes and mixed with beeswax. Bees use propolis for coating the hive to increase strength, i.e. it is also called bee-glue; in blocking holes and cracks in the hive and to adversely affect hive-invading microbes and insects. The chemical constitutents in propolis are related to bud and wound exudates collected and modified by bees from various flora (Burdock, 1998).

The propolis origin reflects the tree populations in any one region: popular (*Populus* spp.), alder (*Alnus glutinosa*), horse chestnut (*Aesculus hippocastanum*), birch (*Betula alba*), beech (*Fagus sylvatica*), and various conifer species (Bankova et al., 2000; Bankova, 2005). In Brazil, propolis samples have been classified into 13 types based on physiochemical characterization with certain propolis types containing profiles similar to the resinous secretions from certain regional flora (Silici and Kutluca, 2005; Daugsch et al., 2007). In general, bee glue is composed of resin and balsam (50%), wax (30%), essential and aromatic oils (10%), pollen (5%) and other substances such as organic material (Burdock, 1998). More than 200 compounds have been identified in different propolis samples (Casaldo and Capasso, 2002; Silici and Kutluca, 2005; Viuda-Martos et al., 2008). Flavonoids, aromatic acids, diterpenic acids and phenolic compounds are thought to be the principle constitutents responsible for the bioactivity of propolis (Viuda-Martos et al., 2008). Propolis has been used in traditional medicine due to its biological properties such as: antibacterial, anticavity, antitumor, antioxidant, antiviral, antiinflammatory, and immunomodulatory effects, among other beneficial attributes (Viuda-Martos et al.,

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2008). Thus, propolis is a rich natural product to explore for novel therapuetic molecules.

In bacteria, the expression of certain bacterial genes, including the virulome or total complement of virulence genes, frequently depends on the cell density of the population. This phenomenon, termed quorum-sensing (QS), is mediated by specific molecules called *N*-acyl-homoserine lactone (AHL) QS signals also known as autoinducers (White and Winans, 2007; Lowry et al., 2008; Atkinson and Williams, 2009). The specificity of AHLs is determined by the acyl side chain length, degree of its saturation and by the presence or lack of an oxo-, or hydroxy-group in the C3 position (Camilli and Bassler, 2006; Scott et al., 2006). AHLs are characterized as long- or short-chain AHLs depending on whether their acyl moiety consist of >8 or ≤8 carbons, respectively. Examples of bacterial phenotypes controlled by QS include biofilm formation, swarming motility, virulence factor expression, bioluminescence and other traits (Lowry et al., 2008; Atkinson and Williams, 2009). In this scenario, the QS mechanism in bacteria enable the regulation of gene expression and coordinated functions beneficial only when carried out by a large number of bacterial cells.

QS plays a intergral role in host-microbial interactions in animals, marine systems and in some organisms that cause plant diseases. Over 68 Gram-negative bacterial species contain at least one complete AHL QS circuit. This includes the QS core proteins known as LuxI-type protein (the AHL synthase), and a LuxR-type protein (the response regulator or receptor) (Case et al., 2008; Atkinson and Williams, 2009). The manipulation of the regulation of QS may provide alternative and novel therapeutic approaches against bacterial pathogens (Rasmussen and Givskov, 2006a,b; Dobretsov et al., 2009). Details on the biochemical and molecular mechanism underlying QS regulation and developing approaches to manipulate QS regulated behaviors in bacteria have recently been investigated (Rice et al., 2005; McDougald et al., 2007; Dobretsov et al., 2009).

One approach to treat antibiotic resistant bacteria is by the development of new mechanisms of antipathogenic treatments that act to attenuate the expression of virulence, which is less likely to impose a selection pressure for development of resistance (Rasmussen and Givskov, 2006a,b; Rasko and Sperandio, 2010). A strategy to develop novel antipathogenic treatments is by blocking the cell-to-cell communication mediated by QS systems. Over the last few years, many natural and synthetic compounds have been identified as QS disruptors (Rasmussen and Givskov, 2006a,b; Dobretsov et al., 2009; Rasko and Sperandio, 2010). The majority of the QS disruptor compounds have been identified and shown to inhibit QS signaling in screens using one response regulator (receptor) and thus may limit the application to a single QS receptor system. A preliminary screening of natural and synthetic libraries for inhibitors revealed that propolis displayed a qualitative inhibitor activity based on a LuxR-dependent system (Rasmussen et al., 2005).

In this work, we investigate the effects of propolis on its ability to agonize or antagonize QS-regulated responses in six AHL-signal-dependent reporter strains, each containing different LuxR-homolog receptor. We show using forward assays that the propolis at concentrations that are not inhibitory to bacterial biosensor(s) growth do not contain AHL signal mimics. However, using reverse assays, propolis inhibits the QS mechanism in agar- and liquid-based assays and in reverse-phase thin-layer chromatography coupled to bioreporter overlaid detection. We show that propolis that differ in their region of origin, physiochemical profile and absorption spectrum exhibit different QS inhibitory responses and that this response depends on the receptor protein.

Moreover, we shown that propolis inhibited swarming motility in the opportunistic pathogen *Pseudomonas aeruginosa* PA01. Propolis also inhibited the AHL-dependent *Pseudomonas aeruginosa* PAO1 LasR and RhlR receptors in biosensor assays paired with near-isogenic strains lacking the corresponding LuxR-type receptor.

2. Materials and methods

2.1. Commercial tincture and raw propolis

Five commercial sources of propolis tincture were used in this study. Propolis A, a 65% extract produced by Natural Factors and a product of Canada. Propolis B, a 50% extract produced by Soria Natural and a product of Spain. Propolis C, a 40% extract produced by Gol-Natur and a product of Italy. Propolis D, a 50% extract produced by Beehive Botanicals and Propolis E, a 70% produced by Y.S. Organic Bee Farms and both products of USA. HFP was extracted in 70% ethanol to produce a tincture (Isla et al., 2005).

2.2. Bacterial strains and growth conditions

Escherichia coli strains JM109 and JLD 271, *Chromobacterium violaceum* CV026, and *Pseudomonas aeruginosa* PA01 were grown in Luria-Bertani (LB) (Winson et al., 1998; Lindsay and Ahmer, 2005; Scott et al., 2006; Shrout et al., 2006; McClean et al., 1997). Each bacterial bioreporter strain used in this work is listed in Table 1 along with its AHL receptor protein and cognate AHL signal. All media were solidified with 16 g of Bacto Agar (Difco) per liter. For strain selection, media were supplemented with the following antibiotics: ampicillin (100 μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml) for *Escherichia coli*.

2.3. Effects of propolis on biosensor growth and the reporter (luciferase) system

An overnight liquid culture of JM109(pSB401) growing in LB was diluted 1:5 three hours before the start of the assay. Each experimental sample contained: 0.0005% (v/v) of the respective propolis (Tincture A–E) placed on sterile cellulose disks, 50 nM 3-O-C6 HSL, and JM109(pSB401) diluted to a starting $OD_{600\,nm}$ = 0.05. In the place of the propolis, the control group contained blank sterile disks. Data for the control and experimental groups represents the average of three replicates. After 0, 3, 6, 9, 12, 21, and 24 h incubation (grown at 30 °C while shaking at 150 rpm) $OD_{600\,nm}$ measurements were made. To evaluate the effects of propolis on the luciferase system, JM109 (pTIM2442), which constitutely produces luciferase at high levels (Alagely et al., 2010) was grown in the presence of the propolis tinctures at 0.0005% (v/v) and relative light units were measured at 0, 5 and 24 of growth using a Turner 20/20 Luminometer at a sensitivity setting of 39.9.

To test the effect of the propolis tinctures on the growth of biosensor *Chromobacterium violaceum* CV026, cellulose discs of 6 mm in diameter were impreganted with propolis tinctures A, B, C, D, and E and place on the surface of a soft-agar plate seeded with strain CV026. Ten microliter of propolis concentrations ranging from full strength to 10^{-4} dilutions of the five tinctures were prepared in 70% EtOH and applied to the discs. After 24 and 48 h of incubation the zone of inhibition as observed as a transparent zone directly adjacent to the disc was measured.

2.4. Inhibition and stimulation of bacterial QS by propolis tincture

We used bacterial reporter strains to test for compounds present in commercial propolis tincture samples. To determine if propolis contains molecules with varying specificity, we first used five different bacterial biosensors that differed by their LuxR homolog receptor (Table 1). The presence of molecules in propolis that stimulate or inhibit QS can be tested in forward and reverse assays using

Table 1
N-acyl-homoserine lactone (AHL) signal bioreporter strains used in this study.

Biosensor strain	AHL receptor	Cognate AHL[a]	Reporter system	Source<s>
Escherichia coli JM109 (pSB401)	LuxR	3-oxo-C6-HSL	*lux*	Winson et al. (1998)
Escherichia coli JM109 (pSB536)	AhyR	C4-HSL	*lux*	Swift et al. (1997)
Escherichia coli JM109 (pSB1075)	LasR	3-oxo-C12-HSL	*lux*	Winson et al. (1998)
Escherichia coli JM109 (pTIM2442)	None	n/a	*lux*	Alagely et al. (2010)
Chromobacterium violaceum CV026	CviR	C6-HSL	violacein	McClean et al. (1997)
Escherichia coli JLD271 (pAL101)	RhlR	C4-HSL	*lux*	Lindsay and Ahmer (2005)
Escherichia coli JLD271 (pAL102)	None	n/a	*lux*	Lindsay and Ahmer (2005)
Escherichia coli JLD271 (pAL105)	LasR	3-oxo-C12-HSL	*lux*	Lindsay and Ahmer (2005)
Escherichia coli JLD271 (pAL106)	None	n/a	*lux*	Lindsay and Ahmer (2005)

[a] C4-HSL, *N*-butanoyl-homoserine lactone; C6-HSL, *N*-hexanoyl-homoserine lactone; 3-oxo-C6-HSL, *N*-3-oxo-hexanoyl-homoserine lactone; 3-oxo-C12-HSL, *N*-oxo-dodecanoyl-homoserine lactone.

the biosensors in the absence and presence of the cognate inducing AHL signal, respectively.

In *Chromobacterium violaceum* strain CV026, the LuxR homolog, CviR regulates the production of a purple pigment, violacein, with exogenous short-chain (C4–C8) alkanoyl or 3-oxo-alkanoyl side chain AHL (forward assays) (McClean et al., 1997; Scott et al., 2006). The QS in CV026 is inhibited by the presence of long-chain *N*-acyl side chain (C10–C14) AHLs, thus blocking violacein production in the presence of the stimulator AHL in reverse assays (C6-HSL and C4-HSL). *Escherichia coli*-based AHL bioreporters used in this work contain plasmid pSB401, pSB1075 or pSB536 (Winson et al., 1998). These reporters in strain JM109 employ different LuxR receptor proteins. For example JM109 (pSB401) contains the LuxR receptor and will produce light in response to exogenous acyl-HSLs via the activation of the *luxCDABE* operon. JM109 (pSB401) detects picomolar amounts of the cognate acyl-HSL, 3-oxo-C6-HSL (*N*-(3-oxo-hexanoyl)-L-HSL), and responds with varying sensitivity to acyl-HSLs with acyl side chains from 4 to 12 carbons in length irrespective of the substitution at the 3 position (Gan et al., 2009). Related JM109 *Escherichia coli*-based sensor strains containing plasmids pSB1075 or pSB536 utilize LasR and AhyR receptor proteins and similarly will activate the *lux* operon in response to certain acyl-HSLs. Two additional sets of *Escherichia coli*-based bioreporters were used based on the RhlR and LasR AHL receptors, native to opportunistic bacterium *Pseudomonas aeruginosa*. Each set contains a near isogenic partner that lacks the corresponding LuxR receptor, which provides a powerful control strain for our bioassays (Lindsay and Ahmer, 2005). All the AHL bioreporter strains used in this work are listed in Table 1.

2.5. Forward and reverse assays using AHL-dependent bacterial biosensor strains

Forward assays evaluate propolis for constituents that agonize or mimic AHL signals, whereas reverse assays test for antagonists or inhibitors to the QS AHL signal system. In the forward assays, propolis samples of various concentrations were impregnated into 6 mm sterile cellulose discs and air dried. In the reverse assays, the pure cognate AHL signal was placed on the bottom of the round bottom tube (12 by 50 mm) and air dried. The propolis sample was applied to cellulose disc, air dried and place into the bottom of the assay tube. Cognate AHL signal for *Escherichia coli* biosensors JM109 (pSB401) was 3-oxo-C6-HSL at 10 nM; for JM109 (pSB536) was C4-HSL at 1 mM; for JM109 (pSB1075) was 3-oxo-C12-HSL at 1 nM, unless otherwise noted. For the *Chromobacterium violaceum* CV026 was C4-HSL was used at 25 mM. The *Chromobacterium violaceum* CV026 reverse assay was carried-out as previously by our laboratory (Scott et al., 2006). For the *Escherichia coli* biosensor, an overnight culture grown in LB plus appropriate antibiotics were diluted 1:10 in LB and 200 μl of the diluted biosensor suspension was added to the round-bottom tubes containing the propolis impregnated disc. The assays were incubated at 30 °C for 6 h with shaking at 200 rpm. Bacterial luminescence was measured using a Turner Design TD 20/20 luminometer. Forward and reverse bioassays with biosensor *Chromobacterium violaceum* CV026 were preformed as described previously (Chamber et al., 2005; Scott et al., 2006; Janssens et al., 2007; Gan et al., 2009; Lowe et al., 2009). Violacein was extracted and quantified using the method of Blosser and Gray (Blosser and Gray, 2000). Mean values of the specific luminescence units and violacein concentrations were obtained with three independent samples. Each experiment was repeated at least twice.

2.6. Chemicals

Purified standards of AHLs were purchased from Quorum Sciences Inc., Coralville, IA and Fluka, Switzerland. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

2.7. Swarming motility bioassay using Pseudomonas aeruginosa PAO1

Swarming motility assay was performed as previously described (Shrout et al., 2006) except for minor changes as follows: Thirty μl of ethanolic propolis extract (A, B, C, D, E or HFP) of a 0.01% dilution was inoculated directly in the center of 0.3% (3 g/1 l agar–agar) LB agar plates in 10 μl increments and the ethanol evaporated. *Pseudomonas aeruginosa* PAO1 (Kornberg) was grown overnight at 28 °C in LB broth and diluted to a starting $OD_{600\,nm}$ = 0.4. Subsequently, serial dilutions in 0.9% NaCl reduced the concentration 10^{-6} and 10 μl was inoculated in the center of the LB agar plates, on the dried propolis extract. After 72 h incubation at 28 °C the largest and smallest diameters in millimeters of the swarming bacteria from each of three replicates were measured. The results display the average of these measurements. Control plates contained dried inoculums of 30 μl 100% ethanol then overlaid with PAO1.

2.8. Thin-layer chromatography with biosensor overlay

Propolis tincture samples were analyzed by C_{18} reverse-phase thin layer chromatography (RP-TLC) in reverse QS signal assays using bacterial biosensor overlay to detect migration spots producing an inhibitory effect. Propolis tincture from 4 to 16 μl were applied in 2 μl volumes and developed as previously decribed (Daugsch et al., 2007). After mobile phase solvent was evaporated, the plates were imaged under UV illumination. The chromatograms were then overlaid with expendentially-phase cultures of CV026 in soft agar at 45 °C supplemented with C4-HSL at 30 nM. The RP-TLC plates were incubated for 24–48 h at 28 °C, photographed and a selected lane was paired with UV image of the same lane for a side-to-side comparison (Shaw et al., 1997; Karamanoli and Lindow, 2006).

2.9. Statistical analysis

Data were statistically analyzed using SAS version 9.1 (SAS Institute Inc., 2004, Cary, NC).

2.10. Abbreviations for N-acyl-homoserine lactones

C4-HSL = *N*-butanoyl-homoserine lactone, C6-HSL = *N*-hexanoyl-homoserine lactone, C8-HSL = *N*-octanoyl-homoserine lactone, C12 = *N*-dodecanoyl homoserine lactone, 3-O-C6-HSL = *N*-3-oxo-hexanoyl-homoserine lactone, 3-O-C12-HSL = *N*-3-oxo-dodecanoyl homoserine lactone.

3. Results

3.1. Effect of propolis on the growth of bioreporters and effect on the luciferase system in presence of propolis

We initially tested AHL-dependent biosensor reporter strains *Escherichia coli* JM109 (pSB401) and *Chromobacterium violaceum* CV026 using propolis tincture- impregnated cellulose discs. JM109 showed zones of inhibition adjacent to discs containing full-strength commercial tincture while CV026 showed with 10 μl of 5% tincture. As a result we used propolis at 1% or less concentrations that do not affect JM109 or CV026 bioreporter growth in the work reported here (data not shown).

We further determined that 0.0005% (v/v) of the five propolis tinctures in growth medium does not affect growth rate or the final growth obtained from the *Escherichia coli*-based biosensor JM109 (pSB401) (Fig. 1A). To test if propolis affects the luciferase activity in AHL-dependent biosensor strains, we used a bioreporter that constitutively expresses the *lux* operon from the phage λ promoter constructed by the Teplitski group (Alagely et al., 2010), JM109 (pTIM2442). JM109 (pTIM2442) in the presence of 0.0005% (v/v) of propolis failed to interfere with luciferase activity when compared the bioreporter in the absence of propolis (Fig. 1B). In general, higher relative light units (RLU) were observed with from JM109 (pSBTIM2442) in the presence of propolis tinctures when compared to the non-propolis supplemented control (Fig. 1B). RLU increased at the 6-hour time and with some tincture samples decreased at the 24-hour determinations. This due to the cultures being in late stationary-phase to early death-phase at 24 h of growth (Fig. 1A and B).

Analysis of the ethanolic propolis tinctures using reversed-phase high performance thin layer chromatography (RPHP-TLC) gave distinct profiles among the five commercial tincture samples initially used in this study (Fig. S1A). Flavonoids are a major component of propolis (Ghisalberti, 1979; Burdock, 1998; Bankova et al., 2000; Bankova, 2005) report demonstrated that different classes of flavonoids can be detected using colorimetric methods (Chang et al., 2002). Since flavonoids will influence the absorption spectra of our individual propolis samples, we performed wavelength scans to discern the differences of the five commerical propolis tinctures. The absorption spectra of the five bee glue tinctures peaked at 250 nm (Fig. S1B). The overall absorption spectra for tinctures A, C, D and E were similar with absorption values reaching below 0.1 between 460 and 500 nm. In contrast, the sample of tincture B showed a shortened tailing effect by reaching an absorption value less that 0.1 at 420 nm (Fig. S1B).

3.2. Effects of propolis tinctures on the activation of the QS regulated phenotypes

Our first set of bacterial biosensor experiments asked whether propolis contained agonist constituents that could regulate bacterial QS activation of the QS-regulated luminescent or violacein

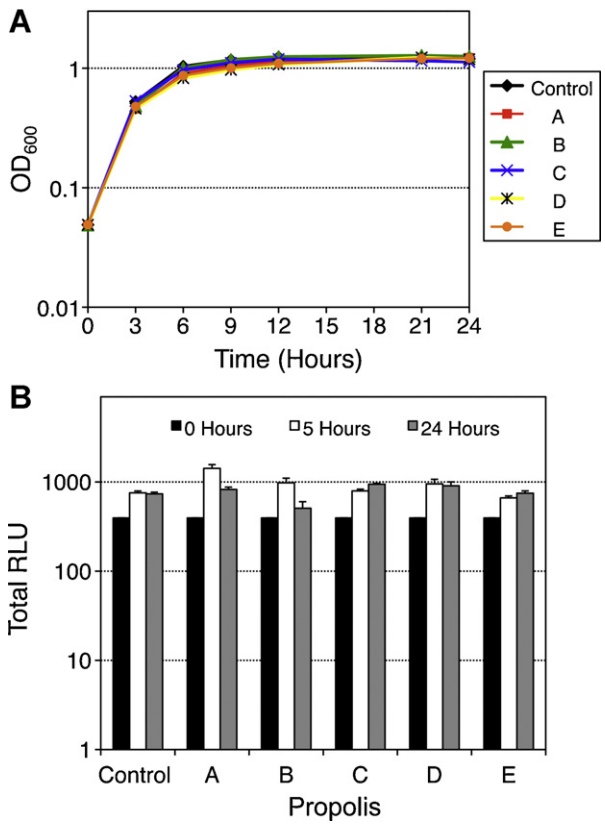

Fig. 1. Propolis tincture do not affect growth of AHL-dependent biosensor strain or the expression of the luciferase system. (A) Growth and accumulation of *Escherichia coli* JM109 (pSB401) in the presence of propolis tinctures A, B, C, D, and E and without propolis (Control). (B) Expression of the constitutely expressed luciferase system in the presence and absence of propolis tinctures A, B, C, D, and E and without propolis at 0, 5 and 24 h of growth. JM109 (pSB401) and JM109 (pTIM2442) was grown in LB medium supplemented with and without propolis tincture and incubated at 37 °C with shaking. Data present is mean ± standard deviation. Abbreviations include: A, B, C, D, and E under the bars in each panel indicate the tincture samples where propolis A is a product of Canada; B is a product of Spain; C, is a product of Italy; D and E, are both products of USA. All experiments were repeated two or more times.

phenotypes (forward assays). Activation of the QS regulated response in all four biosensors was not observed in forward QS assays using propolis tincture between 0.0005% and 0.00000005% in 10-fold serial dilutions (data not shown). This data is consistent with the absence of constitutent(s) in the propolis tinctures that interact with the LuxR receptors in a manner that induces LuxR homolog into the correct confirmation to dimerize and act as a transcriptional activator of the reporter phenotype or output.

3.3. Propolis constitutent(s) antagonize signaling in LuxR-, AhyR- and LasR-dependent biosensors

We investigated whether propolis tincture contained constitutent(s) that disrupt AHL QS signaling systems. Tincture E was able to inhibit quorum sensing most effectively in short-chain AHL biosensor, JM109(pSB401), decreasing reporter activity up to 62% in comparison to the positive control assays (Fig. 2A). Similar decreases in reporter activity is evident with tinctures A, B, C and D in the LuxR-based AHL-dependent biosensor JM109 (pSB401) (Fig. 2A). The AhyR-based reporter was inhibited by the five propolis tinctures up to 38% in comparison to the assays with the addition of AHL only (Fig. 2B). We then asked if propolis tincture contains disruptors of long-chain AHL QS signaling systems? The levels of inhibition for the commercial propolis samples with origins in North America and Europe using JM109 (pSB1075) ranged

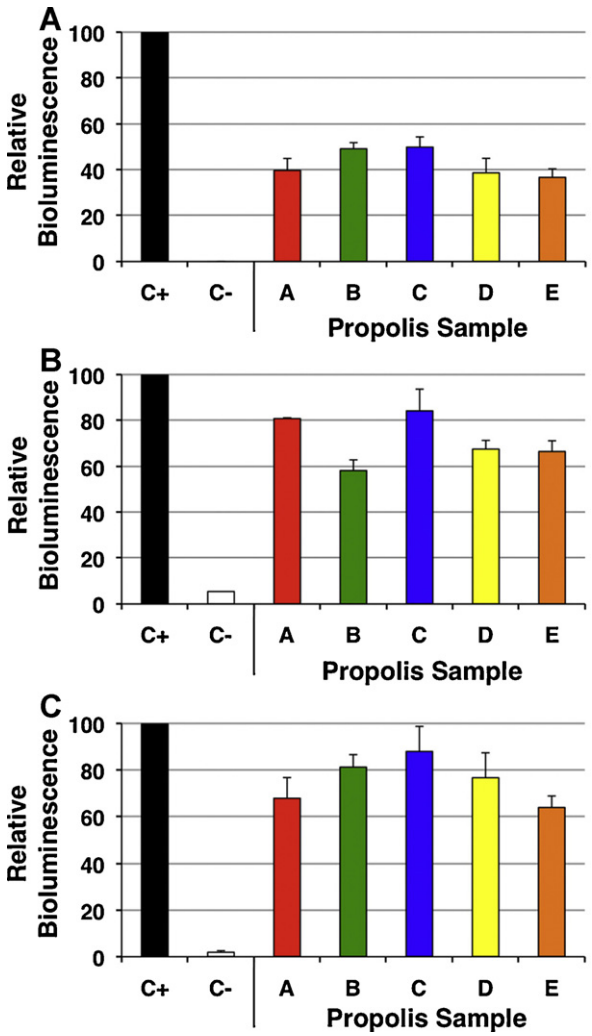

Fig. 2. Inhibition of bioluminescence in short- and long-chain AHL-dependent biosensors by propolis tincture. Relative light units (RLU) were measured after 6 h of incubation in the presence of cognate AHLs and popolis tinctures. Data present is mean ± standard deviation. Effects of propolis tinctures on *Escherichia coli* JM109 (pSB401) AhyR-dependent biosensor (A); on *Escherichia coli* JM109 (pSB536) LuxR-dependent biosensor (B); and on *Escherichia coli* JM109 (pSB1075) LasR-dependent biosensor (C). Abbreviations of A, B, C, D, and E under the bars in each panel indicated the tincture samples where propolis A is a product of Canada; B is a product of Spain; C, is a product of Italy; D and E, are both products of USA. Additional abbreviations under the bars include: positive control, C+, the bioreporter without tincture but with AHL and negative control, C−, without tincture and AHL. The differences among treatments in JM109 (pSB401) are highly significant ($P = 0.012$), for JM109 (pSB401) are significant P = 0.009), and for JM109 (pSB1075) are very significant ($P = 0.0005$). No significant bacterial biosensor growth inhibition, noted by viable CFU counts after 5 h of incubation in the assay, was observed at the concentation of 0.0005% commericial propolis tinctures (data not shown). All experiments were repeated at least twice with representative data of one experiment given.

from 19–42% when compared to the cognate signal only controls (Fig. 2C).

3.4. Concentration-dependent inhibitory activity of QS regulated phenotype

We assested whether propolis tincture can suppress the QS response in a dose-dependent fashion. To test this hypothesis, the QS response was measured in two short-chain AHL indicator strains CV026 and JM109 (pSB401) using varying concentrations of propolis tincture B. Propolis tincture B was selected as a result of its strong inhibitory effects observed using all three *Escherichia coli*-based bioreporters (Fig. 2). C6-HSL induced violacein production in

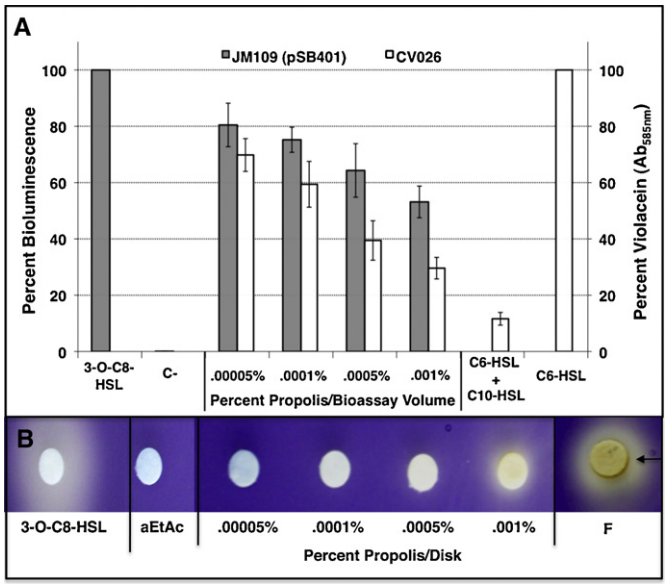

Fig. 3. Concentration-dependent inhibition QS by Spanish propolis. (A) Inhibition of violacein production in CviR-dependent biosensor strain CV026 and bioluminescence in LuxR-dependent biosensor JM109 (pSB401) by propolis B tincture. Violacein production in CV026 was measured after extraction by spectrophotometrically at $A_{585\,nm}$. Bioluminescence production in JM109 (pSB401) was measured with a Turner Designs luminometer at 30% sensitivity. Data present is mean ± standard deviation. The differences among treatments in both JM109(pSB401) and CV026 are highly significant ($P < 0.001$). All experiments were repeated at least twice. (B) Inhibition of AHL-regulated violacein synthesis in CV026 by propolis B using a disc diffusion assay. Short-chain signal C4-HSL (final concentration of 2.42 μM) together with CV026 was added to soft agar plate. Cellulose discs impregnated with propolis B were place on the surface of the seeded agar plate and incubated at 28 °C overnight. Abbreviations include: 3-O-C12-HSL; positive control disc impregnated with long-chain signal 3-O-C12-HSL (2 μl of 1 mM). Controls of pure solvents included: acidified ethyl acetate, aEtAc and ethyl alcohol (data not shown), did not induce a response.

CV026 and bioluminescence in JM109 (pSB401) was inhibited in a dose-response manner with propolis tincture B (Fig. 3A). As a positive control for inhibition of violacein production in CV026 under inducing conditions with C6-HSL, we added long-chain AHL, C10-HSL, to the bioassay and obtained a decrease in pigment production of 87% (Fig. 3A).

To visualize the inhibitor effects of propolis tincture B on the CviR-based biosensor CV026, we tested increasing dilutions of tincture B impregnated in cellulose disk on the AHL-regulated violacein synthesis. Using undiluted tincture growth inhibition (internal zone directly around the disk) (data not shown) was distinguished from the inhibition of the AHL-regulated pigment synthesis (external zone) in CV026 (23) induced with C6-HSL (Fig. 3B). Growth inhibition was apparent as a clear zone around the disk and was caused by adding 10 μl of the undiluted propolis B into the paper disk (data not shown). Beyond the clear or transparent zone was a translucent zone where CV026 growth occurred but violacein production was reduced due to the interference of QS (the outer translucent zone). The presence of two distinct zones suggests that the antimicrobial effects of propolis did not obscure detection of activities that disrupt QS in the propolis tincture. As shown in the serial dilutions of propolis tincture B (Fig. 3B), a reduction in the inhibition of the AHL-induced violacein synthesis is indistinct at dilutions less than the 0.0005% treatment in this bioassay format. The positive control for this assay includes the addition of the long-chain 3-oxo-C12-HSL to a paper disk which results in only translucent zone around the paper disk indicating suppression of AHL-controlled violacein synthesis (Fig. 3B) (Scott et al., 2006).

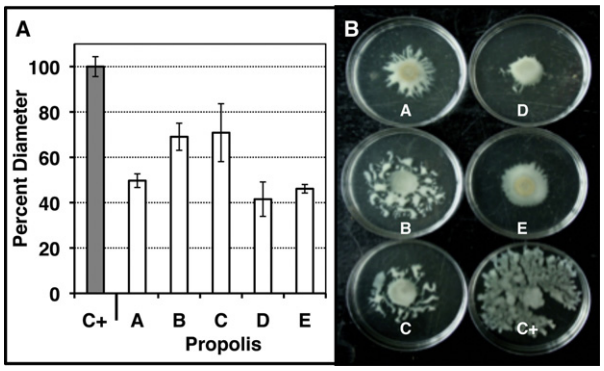

Fig. 4. Inhibition of swarming motility in *Pseudomonas aeruginosa* by propolis tinctures. (A) Average distance in millimeters of swarming motility of *Pseudomonas aeruginosa* PAO1 in the presence of propolis tincture. Data present is mean ± standard deviation. Swarming motility in the presence of the five propolis tinctures were highly significantly different between control (C+) and tincture treatments ($P < 0.001$). (B) Distinct phenotypes of swarming motility in *Pseudomonas aeruginosa* PAO1 in presence of commericial propolis tincture. Panels A, B, C, D and E correspond to propolis origin and without propolis, panel C+. All experiments were repeated three times.

3.5. Inhibition of swarming in opportunistic pathogen, *Pseudomonas aeruginosa PAO1*

Swarming motility has been shown to be regulated by QS in the opportunistic pathogen *Pseudomonas aeruginosa* PA01 (Shrout et al., 2006). Propolis tincture inhibited the QS-regulated cellular function of swarming motility of *Pseudomonas aeruginosa* on 0.3% agar and LB medium. The averages in swarming motility with propolis tincture ranged from 35% to 59% of the control (Fig. 4A). Distinct swarming phenotypes were also identified depending upon the tincture sample (Fig. 4B).

3.6. Hungarian field propolis (HFP) inhibits swarming motility of *Pseudomonas aeruginosa PAO1*

Isogenic sets of *lux* bioreporter plasmids developed in the Ahmer laboratory (Lindsay and Ahmer, 2005) that differ only in the presence or absence of the *luxR* homolog were used. The first set included plasmids pAL101 and pAL102 in the *sdiA-Escherichia coli* strain JLD271. JLD271 (pAL101) contains RhlR, whose cognate signal is C4-HSL; whereas, JLD271 (pAL102) is near isogenic to JLD271 (pAL102) but without RhlR. The second set, JLD271 (pAL105) and JLD271 (pAL106) are with and without the LasR receptor, respectively, and whose cognate signal for LasR is 3-O-C12-HSL. All four bioreporters contain the respective *luxI* promoter fusion to the *luxCDABE* bioluminescent operon as the reporter. These four biosensors are also listed in Table 1.

Further verification of the inhibitory activity of propolis on both *Pseudomonas aeruginosa* swarming motility phenotype and quorum-sensing communication was garnered using a propolis sample collected from a bee hive in Hungary. The fresh Hungarian field propolis (HFP) was extracted in 70/30 ethanol:water (v/v) to produce a tincture. HFP tincture reduced quorum sensing regulated light production in JLD271 (pAL101), the RhlR-based bioreporter, up to 49% without significant effect on light production in near isogenic paired-control bioreporter JLD271(pAL102) (Fig. 5A). In fact, at the lower concentration of propolis, an enhanced metabolic effect was observed as identified by a slight increase in light production in the RhlR-bioreporter (Fig. 5A). This also indicates that HFP at the concentrations used in this work does not affect the growth of these bioreporter strains at the concentration used in these bioassays.

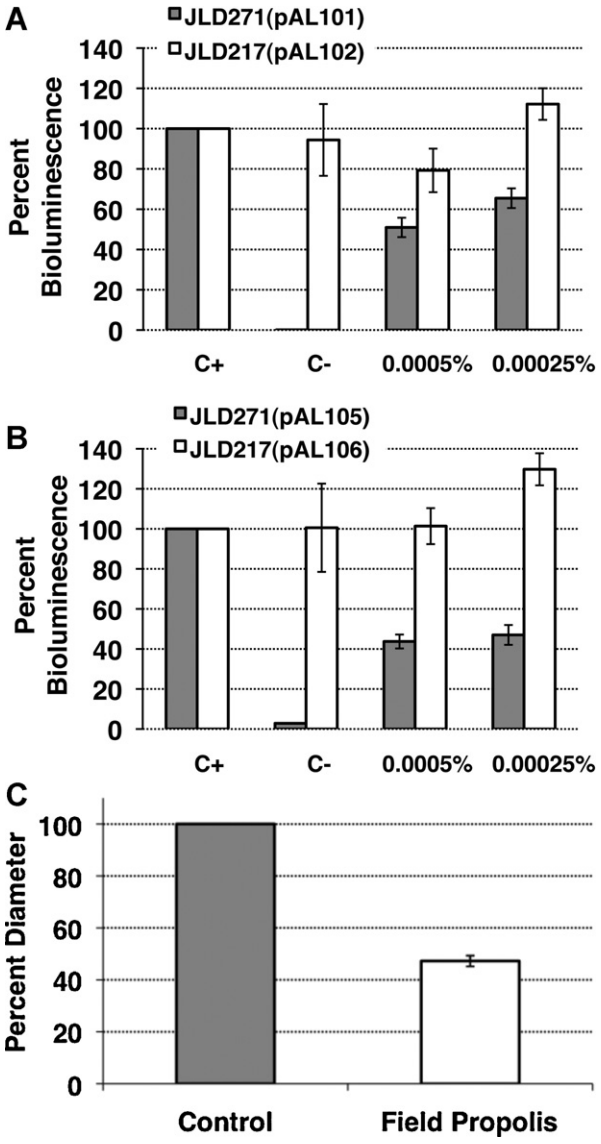

Fig. 5. Effect of Hungarian field propolis (HFP) on *Pseudomonas aeruginosa* LuxR homologs and swarming motility. (A) Relative light units (RLU) by RhlR-dependent bioreporter strain JLD271 (pAL101) and near-isogenic ($RhlR^-$) control bioreporter strain JLD271 (pAL102) in the presence and absence of HFP. (B) RLU by LasR-dependent bioreporter JLD271 (pAL105) and near-isogenic (LasR-) bioreporter JLD271 (pAL106) in the presence and absence of HFP. Data present is mean ± standard deviation. Relative light units (RLU) of RhlR- and LasR-dependent bioreporter strains were highly significantly different among treatments ($P < 0.001$). Isogenic control strains lacking the partner AHL receptor were not significantly different among treatments. (C) Swarming motility in the presence of absence of HFP. Swarming motility was highly significantly different between control and HFP treatements ($P < 0.001$). Each of these experiments were repeated at least two times.

HFP also reduced the quorum sensing phenotype in the LasR-based bioreporter, JLD217 (pAL105), up to 57% without significantly affecting growth and again, at the lower concentration enhanced light production above the control of the near isogenic paired-control bioreporter JLD217 (AL106) (Fig. 5B). Again, this indicates that HFP does not inhibit the growth of the JLD217-based AHL-dependent biosensor strains.

The extracts of HFP were found to inhibit swarming motility by 61% when compared to the control (Fig. 5C). The bioreporter and swarming motility results with HFP are consistent with previous experiments using commercial preparations of propolis tincture (Fig. 4).

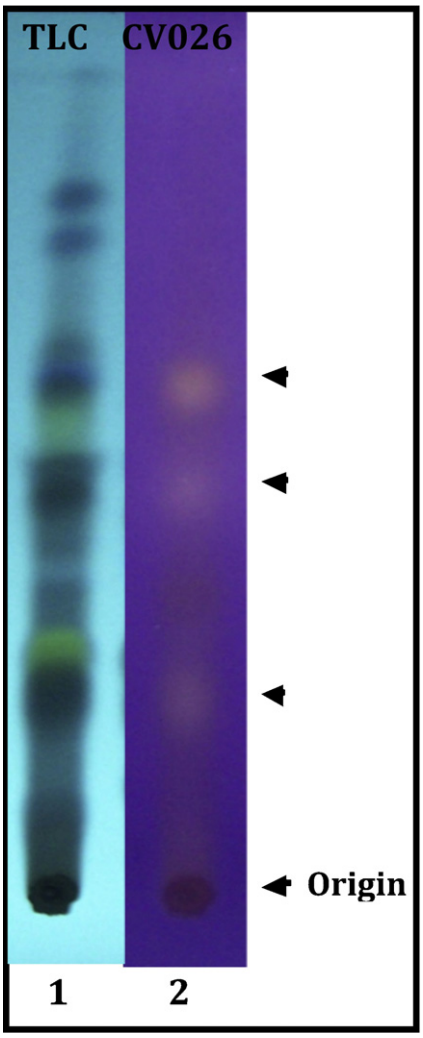

Fig. 6. AHL antagonistic compounds in propolis. AHL antagonistic compounds in propolis tincture after separation and development on a C18 reverse-phase thin-layer plate illustrating the inhibition of C4-HSL-mediated violacein synthesis in bioreporter CV026. Abbreviations include: Reversed-phase high performance (RPHP) thin layer chromatography (TLC) profile of tincture E (lane 1); and RPHP-TLC profile of tincture E after overlaid with the bioreporter CV026 activated with exogenous C4-HSL (lane 2). Areas of pigment clearing (identified by arrows) show regions of tincture E compounds that inhibit AHL-regulated violacein synthesis in *Chromobacterium violaceum* CV026. The experiments were repeated twice.

3.7. Thin-layer chromatography with biosensor overlay

Propolis tincture E was subjected to C_{18} reverse-phase thin layer chromotography and the TLC plate was exposed to ultraviolet (UV) light and photographed (Fig. 6, lane 1), then overlaid with *Chromobacterium violaceum* CV026 in reverse bioassay (Fig. 6, lane 2). A side-by-side comparison of the tincture E profile under UV exposure and with overlaid of CV026 identified three translucent spots in the tincture E profile that inhibited activation of C4-HSL-mediated violacein synthesis (Fig. 6). The presence of translucent spots in the CV026 overlay corresponded with retention factor (Rf) values of UV-identified spots of compounds shown in the RP-TLC plates (Fig. 6).

4. Discussion

Here we provide evidence that bee hive glue or propolis, a natural product, contains constitutents that disrupt AHL QS-controlled systems in bacteria. Compounds that mediate antagonism of AHL QS have been documented from natural sources such as bacteria, fungi, algae and higher plants as well as from synthesis-based compounds with some structures resembling AHL signal molecules (Keshavan et al., 2005; Vattem et al., 2007). However, to our knowledge this is the first quantitative analysis of multiple beehive propolis samples (from North America and Europe) on the cell–cell QS mechanism in bacteria using multiple AHL-dependent bioreporters, in swarming motility assays and by reverse-phase TLC analysis overlaid with an AHL-dependent biosensor in reverse bioassay. In a previous report by Givskov and coworkers, a single qualitative data based on a single LuxR receptor assay showed that a single propolis sample presented an inhibitor activity (Rasmussen et al., 2005). In this work, we confirm and extend that observation with quantitative assays using six different LuxR-type AHL-dependent bioreporters with six geographically distinct propolis samples. We also show that swarming motility in *Pseudomonas aeruginosa* PAO1 and the activation of LasR- and RhlR-regulated bioreporters are also decreased by propolis. Finally, we identified multiple putative QS inhibitory compounds from propolis using reverse-phase TLC with overlay of bioassay. All of these approaches provided data consistent with propolis, a natural product of the beehive, containing constitutents that disrupt AHL QS signaling in bacteria.

QS regulation promotes behaviors that are detrimental to other organisms; thus to protect themselves, some organisms have developed defenses that interfere with, or mimic AHL signals. Over the last decade, workers from various fields have identified natural sources that act as agonists or antagonists to QS regulation (De Nys et al., 1995; Keshavan et al., 2005; Rasmussen and Givskov, 2006a,b; Vattem et al., 2007; Park et al., 2008). The paradigm involves the macroalga *Delisea pulchra* which produces a collection (25–30) of halogenated furanones, many of which inhibit growth of pathogenic bacteria on its surface (Manefield et al., 1999, 2002). The furanones have structural similarity to AHLs and have been shown to inhibit AHL signal from interacting with LuxR-type receptor (Manefield et al., 1999). More recently, it has been demonstrated that rather than displacing the AHL signal from LuxR, the furanones function by increasing LuxR turnover (Manefield et al., 2002). This blocking of AHL signaling by signal displacement or accelerating LuxR degadation, prevents AHL-mediated motility in *Serratia liquefaciens* and inhibits colonization of the alga (Rassmussen et al., 2000). Our studies show that the natural product of the bee hive, propolis, contains components that inhibit AHL-dependent QS regulation in bacteria and thus is a candidate for further anti-QS research aimed at the isolation, identification and characterization of bioactive compounds.

AHLs mimics have been identified in higher plants (Teplitski et al., 2000; Gao et al., 2003) and algae (Teplitski et al., 2004). These discoveries have identified up to 20 chemically separable agonistic as well as antagonistic substances (Teplitski et al., 2000, 2004; Gao et al., 2003). The compounds that affect AHL signaling from plants including pea, crown vetch, rice and the legume model, *Medicago truncatula*, have not been structurally characterized. In addition, it has been shown that *Medicago truncatula* can detect physiological relevant concentrations of AHLs produced by both symbiotic (*Sinorhizobium meliloti*) and pathogenic (*Pseudomonas aeruginosa*) bacteria (Mathesius et al., 2003). *Medicago truncatula*, in response to AHLs, exhibit significant changes in the accumulation of 150 proteins. In addition, exposure to AHLs induces changes in secretion of *Medicago truncatula*-produced AHL-mimic substances (Mathesius et al., 2003). Taken together, certain plants produce AHL agonists and antagonists and can also detect and respond to authentic AHLs by modifying production of the plant's AHL mimics. As a result, plants can significantly influence AHL abundance, composition and perception in associated bacteria. Bees mix plant extracts including resins, waxes, sugars and oils with beeswax which bees secrete

through glands located near their hypopharygeal region of their bodies and β-glucosidase they secrete during plant exudate collection, which results in field propolis found in the hive. Thus, the presence of QS mimics in various plants, and the origin of propolis being derived predominately from plants is consistent that bee glue or propolis contain compounds that can significantly influence QS signaling systems in bacteria.

The marine alga *Laminaria digitata* prevents bacterial surface colonization by producing haloperoxidases that generate oxidized halogens with microbicidal activity. Oxidizing halogens react specifically with AHLs containing an oxygen at the third carbon in the acyl chain and destroy their signaling capability (Borchardt et al., 2001). Likewise, a fungus isolated from marine sponge produce *iso*-cladospolide B and acaterin (Smith et al., 2000) both share structural similarities with furanones, and have been used as chemical structural templates to synthesize QS inhibitory molecules (Hjelmgaard et al., 2003). Intermediates in synthesis of the butenolides from a bryozoan, *Flustra foliacea*, inhibit C6-AHL-regulated phenotype in *Chromobacterium violaceum* (Peters et al., 2003). *Chlamydomonas reinhardtii*, an alga suited for molecular analyses, produces at least a dozen substances that stimulate LuxR homologues in LasR- and CepR-based bacterial biosensors (Teplitski et al., 2004). Further work structurally identified and demonstrated that the vitamin riboflavin and its derivative lumichrome activate the LasR AHL-receptor in bioassays. Amino acid substitutions in the LasR signal binding domain alter the response to both 3-O-C12-HSL, riboflavin and lumichrome (Rajamani et al., 2008). Bacteria, plants and algae commonly secrete riboflavin and lumichrome thus, these compounds can potentially serve as QS signals or interkingdom signal mimics with the ability to manipulate QS in bacteria with a LasR-like receptor. Another anti-AHL-mediated QS strategy has been identified using L-canavanine produced by *Medicago sativa* which interferes with QS signaling in its symbiont, *Sinorhizobium meliloti*, likely through inhibiting the folding of the QS regulatory protein (Keshavan et al., 2005). Consistent with these documented reports are our observations that propolis or bee hive glue, derived in part from plants are likely the origin of the anti-QS bioactive compounds.

Here we observed inhibition of QS in six AHL-dependent bacterial reporter strains with bee hive glue more commonly known as propolis. At this time, the chemical basis of this inhibition is not known, but samples of propolis are chemically complex and varied depending upon the local flora the propolis is collected from by the bees. Consistent with this variation are our observations of different responses by the same biosensor to different propolis tincture samples and to a sample of field propolis. Moreover, we observed differential responses with different biosensors to the same propolis tincture, suggesting that these differences are due to the different LuxR homolog receptors present in bioreporter strains (Figs. 2–4). We tested a library of propolis tincture samples from North America and Europe and a propolis field sample from Hungary at concentrations that do not inhibit the growth of the bioreporter strains and which differ in the LuxR receptor regulating the AHL-dependent reporter phenotype.

The documented use of propolis dates back to at least 300 BC and continues today in topical home remedies and personal products (Burdock, 1998). There is substantial evidence that propolis has antiseptic, antibacterial, antiparasite, antifungal, antiviral, antiinflammatory and antioxidant properties. Our work adds to this long list of beneficial properties of propolis by showing that propolis inhibits a gene-regulatory mechanism in Gram-negative bacteria called quorum sensing. Propolis is an ingredient in toothpaste and dental floss at concentrations around 1% of the finished product (Burdock, 1998). Thus, our observations that tincture and field collected propolis at concentrations less than 1% reduce QS regulated responses in bacteria significantly (in some instances over 50%) is consistent with the use of propolis to reduce cavities, gingivitis, cheilitis and stomatitis. Propolis is also used as a health-food and dietary supplement with recommended dosage of 200 mg per day (Burdock, 1998). To verify that the observed decrease in the QS regulated phenotype in biosensor strains was not caused by growth inhibition or interference on the luciferase system, we monitored the growth of the biosensor strain and the same strain containing a constitutively expressed *lux* operon in the presence of the propolis tincture samples (Fig. 1A and B). These observations taken together strongly suggest that propolis at sub-inhibitory concentrations contains constituents that disrupt autoinducer signaling in bacteria.

Recently microarray analysis in a swarmer motility population of *Pseudomonas aeruginosa* showed that cells from the swarm edge exhibited up-regulation of genes associated with virulence (Tremblay and Deziel, 2010), thus linking between swarming motility and virulence in this opportunistic pathogen. Six different samples of propolis affected motility in *Pseudomonas aeruginosa* swarming assays (Kearns, 2010) and each sample affected motility both qualitatively and quantitatively different. This suggests multiple chemicals or different concentrations but whose bioactivity overlap, and that unique chemicals in each propolis sample exist that influence *Pseudomonas aeruginosa* swarming motility. Alternatively, the swarming motility effects of propolis could be multiple with the involvement of the AHL QS system only part of our observed responses. Other explanations may be that propolis consitituents may affect a more global QS regulator such as GacA (Reimmann et al., 1997). However in our study, consistent with the effect of propolis on *Pseudomonas aeruginosa* motility is the disruption of the *Pseudomonas aeruginosa* LuxR-homologs LasR and RhlR using paired biosensor strains for each LuxR homolog, but with a near-isogenic control biosensor in each pair that lacks the AHL-dependent receptor protein (Lindsay and Ahmer, 2005). Both LasR and RhlR regulate virulence genes, some of which are involved in the regulation of extracellular proteases in *Pseudomonas aeruginosa*.

The distinct propolis profiles after RPHP-TLC separation and identification of putative bioactive compounds via the inhibition of C4-HSL-mediated violacein synthesis with biosensor CV026 by propolis tincture E suggest propolis contains multiple bioactive compounds with a potential to disrupt AHL QS-regulated behaviors (Fig. 6). Further work is needed to identify the compound(s) responsible for AHL QS disruption and determine the mechanism of action provided by propolis.

The nature of the antimicrobial components of propolis has not been completely elucidated although there is evidence they are found among the flavonoids and various esters of caffeic acid. A component active against *Bacillus subtilis* has been identified as 3,5,7-trihydroxyflavone (galangin) (Pepeljnjak et al., 1985; Kosale et al., 2003). On the other hand, it has been suggested that the killing of microorganisms by propolis is the result of synergistic actions of several compounds, none of which alone are particularly effective. It appears likely that the beneficial effects of propolis are the result of their flavonoid content and both natural compounds from propolis samples and purified flavonoids appear to be worthy of further appraisals of their therapeutic efficacy as QS disruptors. Recently such a discovery has been found in extracts from *Combetum albiflorum* bark (Vandeputte et al., 2010). Bark extract from *Combetum albiflorum* contains the flavonoid catechin which was identified as a bioactive molecule that reduces AHL QS-controlled virulence factors in *Pseudomonas aeruginosa* (Vandeputte et al., 2010). The identification of QS disruptors from the natural product propolis may be immensely valuable to further the development of novel antipathogenic treatments by blocking the cell to cell communication mediated by AHL-dependent QS systems. Further work will be needed to identify the bioactive compounds,

either in the synthetic form or by direct purification from propolis samples.

Physiochemical profiles and absorption spectra profiles showed differences in the propolis composition which were further supported by differences in the disruption of the AHL-dependent QS systems in any one biosensor. Likewise, different LuxR receptor homologs in the six biosensor strains responded to different levels of suppression to the same propolis sample. Swarming motility was reduced in *Pseudomonas aeruginosa* PAO1 in the presence of propolis tinctures and field propolis extracts, both consistent with the presence of QS disruptors in bee glue. Using bioreporters carrying the AHL receptors of *Pseudomonas aeruginosa*, RhlR and LasR, a field sample of propolis extracted into a tincture also disrupted the QS-regulated reporter system (bioluminescence) whereas in the near-isogenic bioreporter partners lacking the corresponding LuxR homolog no affect in the bioluminescent output was observed. The characterization of AHL QS antagonistic constituent(s) in bee glue will improve the development of more efficient applications of this natural product and lead to the isolation of the bioactive constituents(s) that inhibit quorum sensing for the development of anti-virulence therapies (Clatworthy et al., 2007). This will enable possibilities for understanding and validating this complex phytotherapeutic as an alternative agent against antibiotic resistant bacterial pathogens (Sforcin and Vankova, 2010).

Author's contributions

This study was conceived, directed and coordinated by M.A.S., who also wrote and critically revised the manuscript with the help of Z.B. and A.O.H. Z.B. participated as an Undergraduate Student in the Biotechnology program at Rochester Institute of Technology during the study by carrying-out the majority of the experiments under the direction of M.A.S. Other experiments were performed by A.O.H. and M.A.S. M.A.S. assisted in the overall data interpretation. P.L. performed statistical analyses on the data sets under the direction of M.A.S. All the authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2011.10.029.

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