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Cinnamon bark oil and its components inhibit biofilm formation and toxin production

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ABSTRACT

The long-term usage of antibiotics has resulted in the evolution of multidrug resistant bacteria, and pathogenic biofilms contribute to reduced susceptibility to antibiotics. In this study, 83 essential oils were initially screened for biofilm inhibition against *Pseudomonas aeruginosa*. Cinnamon bark oil and its main constituent cinnamaldehyde at 0.05% (v/v) markedly inhibited *P. aeruginosa* biofilm formation. Furthermore, cinnamon bark oil and eugenol decreased the production of pyocyanin and 2-heptyl-3-hydroxy-4(1H)-quinolone, the swarming motility, and the hemolytic activity of *P. aeruginosa*. Also, cinnamon bark oil, cinnamaldehyde, and eugenol at 0.01% (v/v) significantly decreased biofilm formation of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC). Transcriptional analysis showed that cinnamon bark oil down-regulated curli genes and Shiga-like toxin gene *stx2* in EHEC. In addition, biodegradable poly(lactic-co-glycolic acid) film incorporating biofilm inhibitors was fabricated and shown to provide efficient biofilm control on solid surfaces. This is the first report that cinnamon bark oil and its components, cinnamaldehyde and eugenol, reduce the production of pyocyanin and PQS, the swarming motility, and the hemolytic activity of *P. aeruginosa*, and inhibit EHEC biofilm formation.

1. Introduction

Many bacteria are able to form biofilms that are attached to various surfaces, such as, polystyrene, glass, stainless steel, in different environments (Goulter et al., 2009; Patel et al., 2011). In clinical and industrial environments these microbial biofilms pose a substantial challenge. In particular, bacterial biofilms are more resistant to conventional antibiotics, host defenses, and contribute to bacterial persistence in chronic infections than cells in suspension (Ceri et al., 1999; Hoffman et al., 2005). Also, pathogenic biofilms are found in medical devices and on food surfaces, and thus, methods of controlling harmful biofilms are urgently required.

Plant-derived essential oils are used as flavoring agents in foods and beverages and their antimicrobial activities make useful natural preservatives. These oils contain potent natural agents that have been used for hundreds of years to combat pathogens, such as, bacteria, fungi, and viruses (Hammer et al., 1999). In previous studies, a number of essential oils, such as, oregano (Lambert et al., 2001), tea tree (Cox et al., 2000), clove (Chaieb et al., 2007), cinnamon, and lemon oil (Friedman et al., 2004) have been found to exhibit antimicrobial effects on both Gram-positive and Gram-negative bacteria.

Pseudomonas aeruginosa is a common nosocomial pathogen and a causative agent of diverse diseases in plants and animals, including humans (Lewenza et al., 2005; Stover et al., 2000). *P. aeruginosa* secretes a large repertoire of virulence factors, such as, quorum sensing molecule PQS (Pseudomonas quinolone signal) (Gallagher et al., 2002), pyocyanin (Lau et al., 2004), rhamnolipids (Zulianello et al., 2006), elastase (Pearson et al., 1997), and two endogenous siderophores, pyoverdine and pyochelin (Michel et al., 2005). It also produces a number of adhesion factors, exotoxin A, phospholipase C (used for hemolysis), and exoenzyme S (Ben Haj Khalifa et al., 2011). In addition, *P. aeruginosa* easily develops antibiotic resistance and is a pathogen of particular importance in cystic fibrosis patients that acquire a lung infection (Cegelski et al., 2008; Stover et al., 2000). Furthermore, its virulence and persistence are generally acknowledged to be associated with biofilm formation (Singh et al., 2000).

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a common human pathogen and has been linked to foodborne illnesses worldwide. EHEC causes bloody diarrhea and possibly life-threatening hemolytic-uremic syndrome by colonizing the large intestine. Furthermore, there is no effective therapy against EHEC infections (Tarr et al., 2005). Therefore, novel non-toxic strategies are required to combat EHEC biofilms. Moreover, unlike cell growth inhibiting antibiotics, biofilm inhibitors

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without bacterial growth inhibition may reduce the emergence of drug resistance (Clatworthy et al., 2007).

The goal of this study was to investigate the antibiofilm activities of 83 essential oils. In order to understand active components and their action mechanism, gas chromatograph/mass spectrometry (GC-MS), microscopic analyses, quorum sensing assays, gene expression assay, and phenotypic assays were utilized. In addition, a biodegradable poly(lactic-co-glycolic acid) (PLGA) surface coating incorporating biofilm inhibitors was prepared and its antibiofilm effects were examined.

2. Materials and methods

2.1. Bacterial strains, essential oils, and growth conditions

All experiments were conducted at 37 °C in Luria-Bertani (LB), which was used to culture *P. aeruginosa* PAO1 (Stover et al., 2000) and *E. coli* O157:H7 (ATCC 43895, EDL933 strain) (Strockbine et al., 1986). To culture *E. coli* O157:H7/pCM18 tagged with green fluorescent protein, LB broth containing 300 μg/ml of erythromycin was used to maintain the pCM18-GFP plasmid, and to culture *P. aeruginosa* PAO1/pMRP9-1 cells tagged with green fluorescent, LB broth containing 150 μg/ml of carbenicillin was used to maintain pMRP9-1-GFP plasmid. Bacterial cells were initially streaked from −80 °C glycerol stock on LB plates. After growth on LB agar plates, cells were cultured from a fresh single colony in LB broth. Then, for phenotypic assays, overnight cultures (stationary phase cells) were inoculated again in LB broth at an initial turbidity of 0.05 at 600 nm. All 83 essential oils (Supplementary Table 1) were obtained from Berjé (Bloomfield, NJ, USA) and Jin Aromatics (Anyang, Gyeonggi Province, Korea). Other chemicals (cinnamaldehyde, eugenol, limonene, linalool, α-pinene, poly(D,L-lactide-co-glycolide), carbenicillin, and erythromycin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For cell growth measurements, turbidity was measured at 620 nm using a spectrophotometer (UV-160, Shimadzu, Japan).

Table 1
GC-MS analysis of cinnamon bark oil. The five components present in the essential oil at greater than 2.5% are indicated by bold font.

No.	SI[a]	RT[b]	Compound[c]	Composition (%)[d]	Identification method[e]
1	**916**	**5.81**	α-Pinene	**3.02**	EI-MS
2	910	6.56	β-Pinene	1.23	EI-MS
3	932	7.38	ρ-Cymene	0.81	EI-MS
4	899	7.46	Limonene	2.53	EI-MS
5	**886**	**8.64**	Linalool	**4.82**	EI-MS
6	902	10.14	α-Terpineol	0.26	EI-MS
7	840	10.53	Benzaldehyde	1.80	EI-MS
8	**823**	**11.43**	Cinnamaldehyde	**64.49**	EI-MS
9	894	11.58	Safrole	0.73	EI-MS
10	**898**	**12.54**	Eugenol	**16.57**	EI-MS
11	816	12.79	α-Copaene	0.12	EI-MS
12	901	13.41	*trans*-Caryophyllene	1.10	EI-MS
13	899	13.62	Cinnamyl acetate	1.15	EI-MS
14	838	13.86	α-Humulene	0.20	EI-MS
15	852	14.62	Acetyleugenol	0.48	EI-MS
16	611	15.49	O-mentha-1(7),8-dien-3-ol	0.20	EI-MS
17	920	17.50	Benzylbenzoate	0.49	EI-MS

[a] SI: Library search purity value.
[b] Retention time (RT).
[c] Compounds are listed in order of elution from a DB-5 capillary column.
[d] Percentage based on FID peak area normalization.
[e] Identification based on computer matching of electron ionization mass spectra using Wiley and NIST libraries for the GC-MS system.

2.2. Crystal-violet static biofilm formation assay

A static biofilm formation assay was performed in 96-well polystyrene plates (SPL Life Sciences, Korea), as previously reported (Lee et al., 2011). Briefly, overnight cultures were inoculated in LB broth (total volume 300 μl) at an initial turbidity of 0.05 at 600 nm and cultured with or without cinnamon bark oil and its components for 24 h without shaking at 37 °C. To quantify total biofilm formation, cell cultures were washed three times with H_2O to remove all not-adhered cells, and biofilms were stained with crystal violet for 20 min and rinsed three times with H_2O, extracted with 95% ethanol, and absorbance was measured at 570 nm. Results are the averages of at least twelve replicate wells.

2.3. Gas chromatograph/mass spectroscopy (GC-MS) analysis

A detailed chemical composition of cinnamon bark oil was obtained using a GC/MS (Jeol JMS 700 mass spectrometer) equipped with an Agilent 6890 N GC DB-5 MS fused silica capillary column (30 m x 0.25 m i.d., film thickness 0.25 μm). The capillary column and temperature conditions for the GC-MS analysis were as previously described (Bajpai et al., 2013). GC-MS was performed using an electron ionization system at 70 eV ionization energy. Helium was used as the carrier gas at a constant flow rate of 1 ml/min. Line temperature of the GC injector and MS transfer line were at 280 °C and 250 °C, respectively. An initial oven temperature of 50 °C was maintained for 2 min, and then increased to 250 °C at a rate of 10 °C/min followed by a holding period at 250 °C for 10 min. Diluted samples (1/100, v/v, in methanol) of volume 1.0 ml were injected manually in split-less mode. The relative percentages of cinnamon bark oil components are expressed as percentages and were calculated by normalizing peak areas. Components were identified using GC retention times on a DB-5 capillary column and by computer matching mass spectra using the Wiley and NIST libraries.

2.4. Confocal laser scanning microscopy

Bacterial cells (*P. aeruginosa* PAO1/pMRP9-1 or *E. coli* O157:H7/pCM18 tagged with green fluorescent protein) were cultured in 96-well polystyrene plates (SPL Life Sciences, Korea) without shaking with or without cinnamon bark oil, cinnamaldehyde, or eugenol. Planktonic cells were removed by washing with PBS three times. Biofilms were visualized by excitation with an Ar laser 488 nm (emission wavelength 500 to 550 nm) under a confocal laser microscope (Nikon Eclipse Ti, Tokyo) using a 20× objective (Kim et al., 2012). Color confocal images were constructed using NIS-Elements C version 3.2 (Nikon eclipse). For each experiment, at least 10 random positions in two independent cultures were chosen for microscopic analysis.

2.5. Scanning electron microscopy (SEM)

SEM was used to observe fimbriae production, as previously described (Lee et al., 2011). A nylon filter was cut into 0.5 × 0.5 cm pieces and placed in 96-well plates with 300 μL of cells of initial turbidity 0.05 at 600 nm. The cells and the nylon filters were incubated together in the presence of cinnamon bark oil, cinnamaldehyde, or eugenol (0.05%) at 37 °C for 24 h without shaking to form biofilm cells. They were then fixed with glutaraldehyde and formaldehyde, and post-fixed in sodium phosphate buffer, osmium, ethanol, and isoamyl acetate. After critical-point drying, they were examined using a S-4100 scanning electron microscope (Hitachi, Japan) at voltage of 15 kV and at magnifications ranging from ×2000 to ×10 000.

2.6. Assays of virulence factors

Since *P. aeruginosa* produces diverse virulence factors, such as, pyochelin, pyocyanin, pyoverdine, rhamnolipid, and 2-heptyl-3-