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In vitro Activity of Four Common Essential Oil Components against Biofilm-producing *Pseudomonas aeruginosa*

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ABSTRACT

Biofilm control has become an area of intense study. The effect of four Essential Oil Components (EOC's) on adherence and biofilms on two pathogenic *Pseudomonas aeruginosa* isolates were investigated in this study. The inhibitory activity was tested on polystyrene flat-bottomed microtitre plates using the Crystal Violet (CV) staining assay. The results showed that this activity was dependent on the terpenes concentration used to treat the adherence and biofilm. Treatment of *Pseudomonas aeruginosa* adherence with eugenol, carveol and carvone (0.5 Minimum Inhibitory Concentration), resulted 60, 45 and 54% inhibition for *P. aeruginosa* (CIP A22) and 69, 65 and 42% for *P. aeruginosa* (ATCC 27853), respectively. β-ionone shows a slight inhibitory effect for the two strains studied. The results also showed that eugenol (0.5 Minimum Inhibitory Concentration) was able to induce an inhibition≥90% of *P. aeruginosa* biofilm (strain ATCC 27853) and a concentration of carvone and carveol of at least 0.5 Minimum Inhibitory Concentration (MIC) was required to obtain approximately 50% of biofilm inhibition. β-ionone (0.5 MIC) appeared as the least efficient against *P. aeruginosa* biofilm resulted only 43% reduction of the biofilm. This study demonstrated the anti-adherence and antibiofilm activity of terpenes and points out the exceptional efficiency of eugenol, carvone and carveol, which could represent candidates in the treatment of *Pseudomonas aeruginosa* biofilm.

Key words: Biofilm, adherence, essential oil components, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative, ubiquitous, free-living bacterial species that is able to survive in a wide variety of environmental extremes (Tambekar *et al*., 2007; Hardalo and Edberg, 2007). Biofilms of *P. aeruginosa* have been widely investigated (O'Toole *et al*., 2000; Trachoo, 2007) and were found to be responsible for bacterial resistance to antibiotics (Farrag, 2001; Drenkard, 2003; Mah *et al*., 2003; Sadovskaya *et al*., 2010). However, in different industrial and medical fields, such as dentistry, drinking water, cooling water, oil recovery, food processing, paper manufacturing, ship hulls and medical implants, biofilms represent a hazardous and costly problem. For these reasons, biofilm control has become an area of intense study. Various

approaches to prevent initial biofilm formation and inhibit growth and development of bacterial biofilms have been described (Jeyasekaran *et al*., 2000; Machado *et al*., 2006; Mayavu *et al*., 2009). The focus has mostly been on the inhibition of bacterial contamination by both physical and chemical intervention (Jeyasekaran *et al*., 2000; Lebert *et al*., 2007; Machado *et al*., 2006). Furthermore, most studies on the antimicrobial activity of plant extracts and essential oils have been restricted to analysis of their bacteriostatic and bactericidal properties. The strong antimicrobial activity of some major components of essential oils, i.e., terpenes, has been described in several studies (Hosseini Jazani *et al*., 2008; Aggarwal *et al*., 2002; Horvath *et al*., 2010; Reichling *et al*., 2009; Talei and Meshkatalsadat, 2007). New assays investigating other potential roles, such as antibiofilm activity have emerged (Braga *et al*., 2008; Sandasi *et al*., 2008; De Carvalho and da Fonseca, 2007; Niu *et al*., 2006; Knowles *et al*., 2005; Gallucci *et al*., 2010). The aim of this study was to investigate whether terpenic derivatives can reduce the development of *P. aeruginosa* adherence and biofilms *in vitro*. This study was carried out using 4 terpenic derivatives that correspond to major components of essential oils: eugenol, β-ionone, caveol and carvon.

MATERIALS AND METHODS

Organisms and growth conditions: Two isolates of *Pseudomonas aeruginosa* were studied. *Pseudomonas aeruginosa* ATCC 27853 was purchased from the American Type Culture Collection. *Pseudomonas aeruginosa* CIP A22 were obtained from (Collection Institut Pasteur, Paris, France). The isolates were revived from glycerol stock cultures kept at -80°C and subcultured onto Luria Bertani (LB) agar plates and incubated at 37°C for 24 h. Prior to use in the adherence and biofilm experiments, the cells were harvested, washed twice in 0.1M (KNO_3) and adjusted to 5×10^6 CFU mL^{-1}.

Minimum inhibitory concentration of terpenes: Four terpenic derivatives (eugenol, β-ionone, caveol and carvon) were purchased from Sigma-Aldrich and were prepared as stock solution of 10% (v/v) in Agar (0.2%) (Remmal *et al*., 1993). The Minimum Inhibitory Concentrations (MIC) of the 4 tested terpenic components were determined by microdilution method previously described by NCCLS (2006).

Effect on adherence and biofilm formation: The effect of different concentrations of the 4 terpenic derivatives (eugenol, β-ionone, caveol and carvon) (ranging from ½ MIC to 1/16 MIC) on adherence and biofilm-forming ability was tested on polystyrene flat-bottomed microtitre plates as described by Cramton *et al*. (1999) with some modifications. Cultures were grown overnight in 10 mL LB, diluted in growth medium to 10^8 CFU mL^{-1} and 100 µL was dispensed into each well of 96 well polystyrene flat-bottomed microtitre plates in the presence of 100 µL of 4 terpenic derivatives (eugenol, β-ionone, caveol and carvon) (1/2 MIC to 1/16 MIC) or 100 µL medium (control). A semi-quantitative measure of the formed biofilms was calculated using a crystal violet assay as described by Nostro *et al*. (2007). Each assay was performed in quadruplicate and repeated at least three times. As a measure of efficacy, relative biofilm formation was defined as follows:

$$\frac{\text{Mean OD492 of treated well}}{\text{Mean OD492 of control well}}\times 100$$

Crystal violet staining assay: Biofilm formation was indirectly assessed using the modified crystal violet assay as described previously (Djordjevic *et al*., 2002). In brief, after incubation for 24 h at 37°C, plates were washed five times with sterile distilled water to remove any loosely associated or planktonic bacteria. The plates were air-dried and then oven-dried at 60°C for 45 min. The wells were then stained with 100 μL of 1% crystal violet and incubated at room temperature for 15 min following by five times wash with sterile distilled water. The semi-quantitative assessment of biofilm formation was performed by adding 200 μL of ethanol to destain the wells. One hundred microlitres from each well was then transferred to a new plate and the absorbance determined at 490 nm. The mean of the triplicate samples and the standard deviations were determined and plotted against EOC incubation time. The antimicrobial effect was measured by comparing the readings of the EOC treated biofilms to a positive and negative control.

Statistical analysis: Statistical analysis was performed using ANOVA. The MINITAB 16 for Windows statistical program was used to determine the mean, standard deviation and evaluate the significance of the data in the tests.

RESULTS AND DISCUSSION

An initial investigation was carried out to assess the efficacy of 4 terpenic derivatives to inhibit the growth of planktonic *Pseudomonas aeruginosa* strains. The results confirmed the potential antibacterial activity of almost all the tested terpenes. MICs ranged between 0.02 and 0.05% (v/v), except for β-ionone that showed MIC (0.5%) (v/v). Thus, we confirmed the weak antimicrobial activity of for β-ionone which had been previously described by Kubo *et al*. (1993). Our results demonstrated the strongest antibacterial activity of eugenol which showed MICs ≤0.02% (v/v), in agreement with observations made by other authors Cox and Markham (2007). It seems reasonable that their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the Proton Motive Force (PMF), electron flow, active transport and coagulation of cell contents (Sikkema *et al*., 1995). Terpenes alter cell permeability by penetrating between the fatty acyl chains making up the membrane lipid bilayers.

It has been previously observed that adherence represents a major step in biofilm formation. The anti-adherence activity of essential oils and terpenes has been poorly studied until now. Therefore, a second investigation was carried out to assess the efficacy of the same 4 terpenes to inhibit *Pseudomonas aeruginosa* adherence. The results of the screening of the inhibitory effects of terpenes on bacterial adherence are shown in Table 1. The objective of this screening test was to exclude the low ECOs which produce more than 50% adherence. This activity was dependent on the terpenes concentration used to treat the adherence. Our study shows that doses of 0.5 MIC produced a greater influence than 0.25, 0.125 and 0.062 MIC. This effect was more evident for *P. aeruginosa* (CIP A22) than for *P. aeruginosa* (ATCC 27853). In the presence of eugenol, carveol and carvone (0.5 MIC), the mean biofilm formation values were equal to 60±3.9, 45±2.2 and 54±2.2% for *P. aeruginosa* (CIP A22) and 69±2.1, 65±2.8 and 42±3.5% for *P. aeruginosa* (ATCC 27853), respectively. β-ionone shows a slight inhibitory effect who represents only 41±3.1 and 37±1.5% for the two strains studied, respectively. Although the mechanism of action of carveol and carvone on adherence remains unclear, the presence of either carveol or carvone influenced the cell properties, namely the composition in fatty acids of the cellular membrane and cell surface hydrophobicity (De Carvalho *et al*., 2005). Furthermore, adhesion of cells to surfaces is facilitated

by the cell surface hydrophobicity (Van Loosdrecht *et al.*, 1987; Yaskovich, 1998; Kos *et al.*, 2003). Hence, we hypothesize that terpenes led to a considerable variation in the hydrophobic behaviour of the cells and therefore influenced the uptake rate of hydrophobic/hydrophilic compounds. Nevertheless, not only is the bacterial membrane affected by the presence of terpenes, but the enzymes involved in their metabolism may also be activated (De Carvalho *et al.*, 2004) are able to change their surface hydrophobicity.

Essential oils are potential sources of novel antibiofilm compounds especially against microorganism's pathogens (Dalleau *et al.*, 2008; Sandasi *et al.*, 2008). Thus, a final investigation was carried out to assess the efficacy of the same 4 terpenes to inhibit *Pseudomonas aeruginosa* biofilm. *In vitro* studies in this work showed that biofilm development was significantly inhibited by ECOs but their effectiveness varied. This activity was dependent on the terpenes concentration used to treat the biofilm. In our study, eugenol oils exhibited strong activity against the *Pseudomonas aeruginosa* biofilm development. Present results are in agreement with the works demonstrated by Niu and Gilbert (2004). For clarity, results were expressed as inhibition percentages of biofilm development (Fig. 1, 2). Four concentrations of eugenol, caveol, β-ionone

Table 1: Influence of 4 terpenes derivates against *Pseudomonas aeruginosa* adherence

		Inhibition of adherence (%)			
P. aeruginosa strains	Agent	0.5 MIC	0.25 MIC	0.125 MIC	0.062 MIC
P. aeruginosa ATCC 27853	Eugenol	69±2.1[a]	67±3.8[a]	58±0.9[a]	53±1.8[a]
	Carvone	42±3.5	38±2.4	35±4.8	24±1.9
	β-ionone	37±1.5[b]	29±1.9[b]	25±3.6[b]	21±2.5[b]
	Carveol	65±2.8[a]	56±2.3	50±3.7	35±1.4
P. aeruginosa CIP A22	Eugenol	60±3.9[a]	58±4.1[a]	39±2.5a	30±2.3[a]
	Carvone	54±2.2	41±1.9	33±1.6	25±1.3
	β-ionone	41±3.1[b]	34±2.7	30±2.4[b]	18±2.0[b]
	Carveol	45±2.2	32±1.8[b]	29±0.7[b]	21±3.7

The data represent the average and standard deviation of two independent experiments carried with six replicates. Different letters show significent difference within column at p>0.05

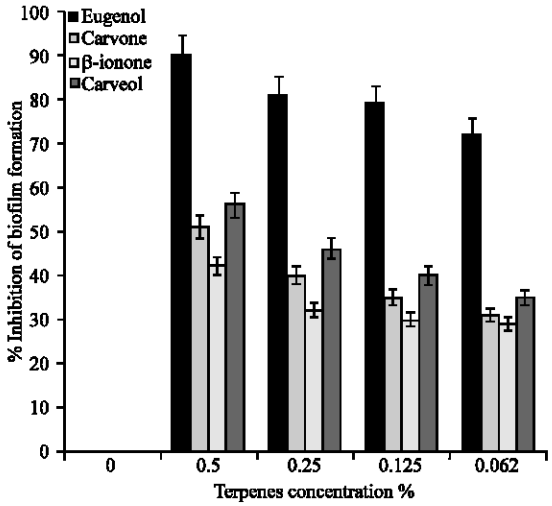

Fig. 1: Influence of 4 terpenes derivates on the biofilm development of *Pseudomonas aeruginosa* ATCC27853

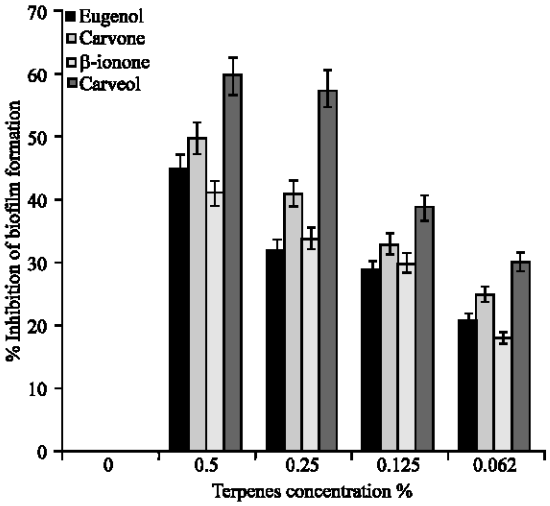

Fig. 2: Influence of 4 terpenes derivates on the biofilm development of *Pseudomonas aeruginosa* CIPA22

and carvon were tested (0.2, 0.25, 0.125 and 0.062%) on biofilms aged 1 old. In these conditions, eugenol (0.5 MIC) was able to induce an inhibition≥90±3.1% of *P. aeruginosa* biofilm (strain ATCC 27853) (Fig. 1). In the same conditions, our results showed that a concentration of carvone and carveol of at least 0.5 MIC was required to obtain approximately 50±1.6% of biofilm inhibition. Finally, β-ionone appeared as the least efficient against *P. aeruginosa* biofilm, because we observed that a concentration of at least 0.5 MIC was necessary to obtain only 43% reduction of the biofilm (Fig. 2). One of the reasons for the low activity was the production of large quantities of exopolysaccharide in *P.aeruginosa* that protects bacterium from bactericide effects of ECOs. Stewart and Costerton (2001) reported that bacterial cells are much more protected from antimicrobial agents when embedded in biofilms cells. This protection can be due to a combination of mechanisms, e.g., reduced diffusion of biocides due to the exopolymeric matrix, physiological changes in the cells, etc. Similar observations regarding the antibiofilm activity of eugenol, β-ionone, carveol and carvon were made using other strain *P. aeruginosa* CIP A22 (Fig. 2). Interestingly, the advantage of using compounds such as carveol and carvone lies in the fact that such compounds, contrarily to antibiotics, affect biofilm formation/destruction without creating a selective pressure that would lead to the appearance of resistant mutants (Fux *et al.*, 2003; Schachter, 2003).

CONCLUSION

Our results pointed out the *in vitro* potential of four terpenes as antibiofilm agents and clearly demonstrate the feasibility of using natural compounds such as terpenes to prevent biofilm formation. Almost all the studied terpenic derivatives showed antibacterial and antibiofilm activity against *Pseudomonas aeruginosa*. However, eugenol was the most efficient in reducing the development of *Pseudomonas aeruginosa* growing planktonically and as adherence and a biofilm.

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