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Full Length Research Paper

Carvacrol and thymol components inhibiting *Pseudomonas aeruginosa* adherence and biofilm formation

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***Pseudomonas aeruginosa* has a high propensity to develop biofilms that are resistant to exogenous deleterious agents. The aim of this study was to investigate whether carvacrol and thymol can interfere with adherence phenomena as well as acting on biofilm formation. Tests of *P. aeruginosa strains* showed that carvacrol and thymol interferes with the starting phases of adherence as well as with *P. aeruginosa* biofilms. Carvacrol and thymol (2MIC) inhibition was 97±8.5 and 89±6.3% for *P. aeruginosa* (ATCC 27853) and 72±4.6 and 69±6.8% for *P. aeruginosa* (CIP A22) adherence respectively. Carvacrol (2MIC) inhibition exceeds 90% for *P. aeruginosa* (ATCC 27853) and *P. aeruginosa* (IL5) biofilm. Thymol (2MIC) inhibition is 86±2.1, 54±5.9 and 70±4.3% for *P. aeruginosa* (ATCC 27853) *P. aeruginosa* (CIP A22), *P. aeruginosa* (IL5), respectively.**

Key words: Biofilm, adherence, carvacrol, thymol, *Pseudomonas aeruginosa.*

INTRODUCTION

P. aeruginosa is a ubiquitous Gram-negative micro-organism found in many environments, such as soil and water. *P. aeruginosa* is also an opportunistic pathogen implicated in respiratory infections, urinary tract infections, gastrointestinal infections, keratitis and otitis media. *P. aeruginosa* is found in an estimated 10–20% of all hospital-acquired infections (Ikeno et al., 2007).

Biofilms are multicellular matrices of bacteria surrounded by extracellular polysaccharides called a glycocalyx. The glycocalyx acts as a physical barrier and is strongly anionic thereby protecting the bacterial microcolony from external agents (Jeyasekaran et al., 2000). Once established, biofilms appear to have greater resistance to exogenous deleterious agents such as antibiotics, detergents or biocides than their planctonic counterparts (Lewis, 2002; Mah and O'Toole, 2001; Stewart and Costerton, 2001). Many approaches have been proposed to inhibit bacterial biofilm formation, including material surface coating (Raulio et al., 2005; Niemela et al., 2005); application of antimicrobial compounds (Tuttlebee et al., 2002); physical methods (Carmen et al., 2004); and enzymatic degradation by bacteriophage (Hughes et al., 1998).

Essential oils and some essential oil components (EOC's) are known to exhibit antimicrobial properties (Inouye et al., 2001). The strong antimicrobial activity of some major components of essential oils, i.e. terpenes, has been described in several studies (Dorman et al., 2002; Ben Arfa et al., 2006). Carvacrol (2-methyl-5-(1-methylethyl) phenol) and thymol (2-isopropyl-5-methylphenol) are known for their wide spectrum of

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antimicrobial activity, which has been the subject of several investigations *in vitro* (Dorman et al., 2002; Lambert et al., 2001) and *in vivo* (Manohar et al., 2001; Adam et al., 1998). In contrast, their antibiofilm activity has not been studied extensively. Thus, we purpose in this study to investigate the effect of carvacrol and thymol essential oil components against *P. aeruginosa* adherence and biofilm formation on polystyrene. The selection of these components was based on their frequent occurrence in aromatic plants.

MATERIALS AND METHODS

Essential oil component and *Pseudomonas* strains

Carvacrol (≥97.0% pure) and thymol (≥99.0% pure) was purchased from Sigma-Aldrich. *P. aeruginosa* (CIP A22) were obtained from Collection Institute Pasteur, Paris, France, *P. aeruginosa* (ATCC 27853) was purchased from the American Type Culture Collection and *P. aeruginosa (IL5)* were isolated from water in our laboratory. All strains were revived from glycerol stock cultures kept at -80℃. The strains were sub-cultured onto Luria Bertani (LB) agar plates and incubated at 37℃ for 24 h. Following incubation, single colonies were transferred from the plates and inoculated into Erlenmeyer flasks containing 25 ml of sterile Luria Bertani (LB) broth. The cultures were incubated at 37℃ for 24 h before the absorbance was read at 600 nm using a spectrophotometer and standardized to 10^6-10^7CFU/ml.

Efficacy of carvacrol and thymol on planktonic cells

The MICs of carvacrol and thymol on planktonic cells were determined using a broth dilution micro-method on polystyrene flat-bottomed microtiter plates previously described by National committee for clinical Laboratory Standards (NCCLS, 2006). The data from at least five replicates were evaluated and modal results were calculated. Stock solutions of 50% (v/v) essential oils components were prepared in agar 0.2% and used following dilution (Remmal et al., 1993).

Effect on adherence and biofilm formation

The effect of different concentrations of carvacrol and thymol (ranging from 2MIC 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 c.f.u/ml and 100 µl was dispensed into each well of 96 well polystyrene flat-bottomed microtitre plates in the presence of 100 µl of carvacrol and thymol (2MIC 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 below. Each assay was performed in quadruplicate and repeated at least three times. As a measure of efficacy, relative biofilm formation was defined as follows: (mean OD_{492} of treated well/mean OD_{492} of control well) ×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℃, 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℃ 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 550 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

Recently, scientific interest in the biological properties of carvacrol and thymol has increased remarkably (Ben Arfa et al., 2006; Chami et al., 2005; Dorman et al., 2002; Kordali et al., 2008; Lambert et al., 2001; Nostro et al., 2004; Ultee et al., 1998). In this study, we initially assessed the efficacy carvacrol and thymol on inhibiting the growth of planctonic *P. aeruginosa* strains. The MICs of both EOC's carvacrol and thymol ranged between 0.02 and 0.05 respectively, thus confirming the antibacterial potential previously described by several authors (Lambert et al., 2001; Cox et al., 2007). Several mechanisms have been proposed to explain the antimicrobial activity of carvacrol and thymol on bacteria. Specifically, carvacrol and thymol can disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane. The presence of magnesium chloride has no influence on this action, suggesting a mechanism other than chelation (Helander et al., 1998).

Adherence represents a major step in biofilm formation. Therefore, a second investigation was carried out to assess the efficacy of the both EOC's (carvacrol and thymol) to inhibit *P. aeruginosa* adherence. A different inhibitory effect among the strains in the presence of concentrations (2MIC to MIC/16) was observed (Table 1). Our study shows that this activity was dependent on the terpenes concentration used to treat the adherence. In the presence of carvacrol and thymol (2MIC), the mean adherence values were equal to 97±8.5 and 89±6.3 % for *P. aeruginosa* (ATCC 27853) and 72±4.6 and 69±6.8% for *P. aeruginosa* (CIP A22). This effect was more evident for *P. aeruginosa* (ATCC 27853) than *P. aeruginosa* (CIP A22) strains.

Despite their broad antimicrobial spectrum, the application of carvacrol and other essential oils in food

Table 1. Influence of thymol (T) and carvacrol (C) against *Pseudomonas aeruginosa* adherence. The data represent the average and standard deviation of two independent experiments carried with six replicates.

P. aeruginosa strains		Inhibition of adherence (%)					
		2MIC	1MIC	1/2MIC	1/4MIC	1/8MIC	1/16MIC
P. aeruginosa ATCC 27853	T	89±6.3	88±4.1	75±3.2	72±7.5	69±3.7	63±5.8
	C	97±8.5	91±3.2	87±6.5	74±3.1	45±2.4	31±4.2
P. aeruginosa CIP A22	T	69±6.8	54±3.4	49±5.8	29±2.3	9±6.0	6.2±1.9
	C	72±4.6	63±5.3	57±4.2	43±7.6	30±5.9	19±2.4

Table 2. Effects of thymol (T) and carvacrol (C) on *Pseudomonas aeruginosa* biofilms formation at 24 h.

P. aeruginosa strains		Inhibition of biofilm development (%)					
		2MIC	1MIC	1/2MIC	1/4MIC	1/8MIC	1/16MIC
P. aeruginosa ATCC 27853	T	86±2.1	78±3.5	75±3.0	72±2.4	69±5.3	63±2.2
	C	91±0.8	87±1.9	76±5.1	54±4.6	32±2.9	15±3.2
P. aeruginosa CIP A22	T	54±5.9	49±3.1	41±2.3	28±5.2	18±6.3	8.4±9.0
	C	62±1.2	60±2.2	52±2.4	33±4.4	20±3.2	9±5.0
P. aeruginosa IL5	T	70±4.3	61±2.8	30±4.3	20±2.1	12±3.2	8.4±7.9
	C	90±2.5	67±3.6	50±8.4	18±5.2	13±1.7	10±2.7

preservation has been limited by their potent aromatic properties (Roller, 1995). However, their potential for use in cleaning, disinfection, and biofilm control has not been studied extensively. Only a small proportion has focused on its effects on bacterial biofilm. Knowles and Roller (2001) first reported the treatment of bacterial dried films with carvacrol on stainless-steel surfaces. Interestingly, thymol also demonstrated the antibiofilm activity against *Candida albicans* biofilm (Braga et al., 2008). He reported that the metabolic activity of sessile cells was reduced by >90% at twice the minimum inhibitory concentration of planktonic cells.

Finally, we evaluated the activity carvacrol and thymol on inhibiting *P. aeruginosa* biofilm formation. A general attenuated level of biofilm formation in the presence of carvacrol and thymol was observed (Table 2). Doses of 2 MIC produced a greater influence than doses of 0.25 and 0.06 MIC. In the presence of carvacrol and thymol (2 MIC), the mean biofilm formation values were equal to 91±0.8 and 86±2.1% for *P. aeruginosa* (ATCC 27853), and 62±1.2% and 54±5.9% for *P. aeruginosa* (CIP A22), and 90±2.5% and 70±4.3 for *P. aeruginosa (IL5)*, respectively. Carvacrol and thymol have a log Po/w of 3.64 and 3.30, respectively (Griffin et al., 1999; Ultee et al., 2002). Weber and de Bont (1996) reported that compounds with a log Po/w value higher than 3 are highly potent on disturbing cell membrane. However, carvacrol and thymol have been reported to possess a relative hydrophilicity of 830±10 and 846±9 p.p.m., respectively (Griffin et al., 1999). Although the mechanism of action of Carvacrol and thymol on biofilms remains unclear, their amphipathic nature could account for the observed effects. Hence, we hypothesize that the relative hydrophilicity of carvacrol and thymol may allow their diffusion through the polar polysaccharide matrix, whilst the prevalent hydrophobic properties of these compounds could lead to specific interactions with the bacterial membrane. This hypothesis is supported by the anti-plaque effects of a thymol-based mouthwash, in part attributable to rapid-kill and plaque-permeating abilities (Ouhayoun, 2003).

We observe also that *P. aeruginosa* are much more protected from carvacrol and thymol components when embedded in biofilms. Similar results have been observed by Stewart and Costerton (2001). They reported that protection from antimicrobial agents in biofilms is due to a combination of mechanisms, e.g. reduced diffusion of biocides due to the exopolymeric matrix, physiological changes in the cells due to reduced growth rates and production of enzymes able to degrade the antimicrobial substances.

In conclusion, the findings of the present study highlight the promising role of carvacrol and thymol as new lead structure in the search for anti-adherence and antibiofilm agents.

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