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Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms

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The aim of this study was to evaluate the effect of oregano essential oil, carvacrol and thymol on biofilm-grown *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, as well as the effects of the oils on biofilm formation. For most of the *S. aureus* (n=6) and *S. epidermidis* (n=6) strains tested, the biofilm inhibitory concentration (0.125–0.500 %, v/v, for oregano, and 0.031–0.125 %, v/v, for carvacrol and thymol) and biofilm eradication concentration (0.25–1.0 %, v/v, for oregano and 0.125–0.500 %, v/v, for carvacrol and thymol) values were twofold or fourfold greater than the concentration required to inhibit planktonic growth. Subinhibitory concentrations of the oils attenuated biofilm formation of *S. aureus* and *S. epidermidis* strains on polystyrene microtitre plates.

INTRODUCTION

Staphylococci are important nosocomial pathogens. Eradication of these micro-organisms is not always successful due to their ability to form biofilms. Experimental evidence has shown that micro-organisms in biofilms are less susceptible to conventional treatment (Brown & Gilbert, 1993) than their planktonic counterparts. Many factors contribute to the lowered susceptibility of bacteria enclosed in a biofilm, and include the induction of a biofilm phenotype, the stress response and failure of the antimicrobial agents to penetrate the biofilm (Mah & O'Toole, 2001).

As such, alternative strategies or more effective agents exhibiting activity against biofilm-producing micro-organisms are of great interest. Natural drugs could represent an interesting approach to limit the emergence and the spread of these organisms, which currently are difficult to treat. Recently, there has been considerable interest in the study of plant materials as sources of new compounds for processing into therapeutic agents. One approach may be the use of essential oils that have been shown to be potential agents in the treatment of infections, and are safe in terms of human and animal health. In this context, oregano oil and its major phenolic components, carvacrol [2-methyl-5-(1-methylethyl)phenol] and thymol (2-isopropyl-5-methylphenol), are known for their wide spectrum of antimicrobial activity, which has been the subject of several investigations *in vitro* (Dorman & Deans, 2000; Lambert *et al.*, 2001) and *in vivo* (Adam *et al.*, 1998; Manohar *et al.*, 2001). They possess multiple biological properties such as anti-inflammatory, anti-leishmanial, antioxidant, hepatoprotective and anti-tumoral activities (Aeschbach *et al.*, 1994; Alam *et al.*, 1999; Robledo *et al.*, 2005; Skold *et al.*, 1998; Weber & de Bont, 1996; Zeytinoglu *et al.*, 2003).

Previously, we have shown the efficacy of oregano oil, carvacrol and thymol against planktonic *Staphylococcus aureus* and *Staphylococcus epidermidis*, including meticillin-resistant strains (Nostro *et al.*, 2004). The objective of this study was to extend the research to evaluate the activity of oregano oil, carvacrol and thymol on biofilm-grown *S. aureus* and *S. epidermidis* strains, as well as the effects of oils on biofilm formation.

Abbreviations: BEC, biofilm eradication concentration; BIC, biofilm inhibitory concentration; MBC, minimum bactericidal concentration.

METHODS

Essential oils. The aerial parts of commercial *Origanum vulgare* L. (obtained from A. Minardi, Ravenna, Italy) were subjected to hydrodistillation. The qualitative and quantitative composition of the essential oil was analysed by GC and GC/electron impact MS as

described previously (Nostro *et al.*, 2004). The oregano oil was characterized principally by carvacrol and thymol (14 and 24.7 %, v/v, respectively) and by their two precursors, γ-terpinene and *p*-cymene (11.7 and 14.6 %, v/v, respectively). Carvacrol ($\geqslant$97.0 % pure) and thymol ($\geqslant$99.0 % pure) were purchased from Aldrich. Stock solutions of 50 % (v/v) essential oils were prepared in ethanol (EtOH) and used following dilution.

Bacterial strains. The bacteria used were *S. aureus* (n=6) and *S. epidermidis* (n=5) isolated from ocular infections belonging to our private collection, and the reference strain *S. epidermidis* ATCC 35984 (slime producing). Each isolate was characterized for biofilm-related properties as reported previously (Blanco *et al.*, 2005). The isolates were capable of forming biofilms with an OD_{492} ranging from 0.52 to 1.55 (Blanco *et al.*, 2005).

Efficacy of oregano oil on planktonic cells. The MIC and minimum bactericidal concentration (MBC) of oregano, carvacrol and thymol on planktonic cells were determined in tryptic soy broth (TSB) using a broth dilution micromethod in polystyrene flat-bottomed microtitre plates (Costar Corning) according to CLSI guidelines (Clinical Laboratory Standards Institute, 2000). The data from at least five replicates were evaluated and modal results were calculated. Two growth controls consisting of TSB medium and TSB_{EtOH} were included

Effect on established biofilms. The effect on established biofilms was verified as described by Johnson *et al.* (2002) with some modifications. All isolates were grown as biofilms using polystyrene flat-bottomed microtitre plates. After 24 h of incubation at 37 °C, the planktonic-phase cells were gently removed and the wells were washed three times with PBS and filled with 200 µl twofold dilutions of the oils, ranging from the MIC to a 16-fold dilution of the MIC. The plates were incubated for 24 h at 37 °C. The OD_{492} was measured at time 0 and after incubation for 24 h. The biofilm inhibitory concentration (BIC) was determined as the lowest concentration where no growth occurred in the supernatant fluid, confirmed by no increase in optical density compared with the initial reading. Samples of biofilms from the bottom of these wells were scarified by a metal loop, spread over the surface of tryptic soy agar (TSA) plates and incubated for 72 h at 37 °C. The biofilm eradication concentration (BEC) was determined as the lowest concentration at which no bacterial growth occurred on the TSA plates. Data from at least five replicates were evaluated and modal results were calculated. Two biofilm controls consisting of TSB medium and TSB_{EtOH} were included.

Effect on biofilm formation. The effect of different concentrations of oil (ranging from 0.5 to 0.125 MIC) on 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 TSB with 1 % glucose, diluted in growth medium to 5×10^5 c.f.u. ml^{-1} and 100 µl was dispensed into each well of 96-well polystyrene flat-bottomed microtitre plates in the presence of 100 µl subinhibitory concentrations (subMIC) of oregano, carvacrol and thymol (0.5, 0.25 and 0.125 MIC) or 100 µl medium (control). After incubation for 24 h at 37 °C, each well was washed twice with sterile PBS (pH 7.4), dried, stained for 1 min with 0.1 % safranin and washed with water. The stained biofilms were resuspended in 200 µl PBS and OD_{492} was measured by spectrophotometry using an ELISA reader. Two biofilm controls consisting of TSB medium and TSB_{EtOH} were included. 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) $\times$ 100.

Scanning electron microscopy. Biofilms of a strong biofilm producer (*S. aureus* 815) formed on polystyrene flat-bottomed microtitre plates in the presence or absence of subMIC concentrations of carvacrol (0.5 and 0.25 MIC) were fixed in 2 % glutaraldehyde in 0.1 M PBS for 2 h at 4 °C and then post-fixed for 1 h at 4 °C in 1 % osmium tetroxide in the same buffer. After thorough washing with PBS, samples were dehydrated in a series of ethanol solutions (30–100 %). Specimens were mounted on aluminium stubs with conductive carbon cement, allowed to dry and then coated with a gold film. Samples were observed with an S-400 scanning electron microscope (Hitachi).

Statistical analysis. The biofilm formation values were analysed by a hierarchical analysis of variance test following angular transformation. The differences between groups (different oil concentrations) were considered significant when $P<0.05$. Where significant differences existed, the Duncan test was performed to verify the significant difference levels.

RESULTS AND DISCUSSION

In this study, the *in vitro* effects of *O. vulgare* essential oil, carvacrol and thymol on staphylococcal biofilms were evaluated. Remarkably, the *in vitro* activity of the oils on biofilm was only slightly lower than that on planktonic culture (Table 1). For most of the strains tested, the BIC (0.125–0.500 %, v/v, for oregano, and 0.031–0.125 %, v/v, for carvacrol and thymol) and BEC (0.25–1.0 %, v/v, for oregano, and 0.125–0.500 %, v/v, for carvacrol and thymol) values were twofold or fourfold greater than the concentration required to inhibit growth in suspension.

Despite a different inhibitory effect among the strains, a general attenuated level of biofilm formation in the presence of subinhibitory concentrations of oregano, carvacrol and thymol was observed (Table 2). Significant differences ($P<0.05$) in biofilm formation values were observed between groups (different oil concentrations). Doses of 0.5 MIC produced a greater influence than doses of 0.25 and 0.125 MIC (Duncan test). This effect was more evident for *S. aureus* than for *S. epidermidis* strains. In the presence of oregano, carvacrol and thymol (0.5 MIC), the mean biofilm formation values were equal to 46.7, 28.3 and 30.1 % for *S. aureus*, and 58.9, 57.1 and 54.4 % for *S. epidermidis*, respectively. Oregano showed a slight inhibitory effect because carvacrol and thymol represent only a fraction (38.7 %) of the entire essence and they interact in an additive rather than a synergistic way (Lambert *et al.*, 2001).

Direct observation by scanning electron microscopy of *S. aureus* 815 showed that, after 24 h, in the absence of carvacrol (Fig. 1a, b), bacterial cells formed evident biofilms with matrix material. In the presence of carvacrol at concentrations of 0.5 and 0.25 MIC (Fig. 1c, d, e), bacterial cells grew as looser colonies, and the amount of biofilm was reduced, being almost absent at 0.5 MIC.

Having more-effective antimicrobial agents that are also active against biofilm, and are able to prevent or at least interfere with biofilm formation, would be a considerable achievement. As an extension of our earlier work on the efficacy of oregano, carvacrol and thymol against planktonic meticillin-resistant staphylococci (Nostro *et al.*,

Table 1. Susceptibility of planktonic and biofilm organisms to oregano (O), carvacrol (C) and thymol (T)

Strain	Agent	MIC (%, v/v)	BIC (%, v/v)	MBC (%, v/v)	BEC (%, v/v)
S. aureus					
6ME	O	0.062	0.250	0.125	0.500
	C	0.015	0.062	0.062	0.250
	T	0.031	0.062	0.062	0.250
810 CT	O	0.125	0.500	0.250	0.500
	C	0.031	0.125	0.062	0.250
	T	0.031	0.062	0.062	0.125
815 CT	O	0.062	0.250	0.125	0.500
	C	0.031	0.125	0.125	0.500
	T	0.031	0.062	0.062	0.125
808 CT	O	0.125	0.250	0.125	1
	C	0.031	0.125	0.062	0.250
	T	0.031	0.062	0.062	0.125
5 ME	O	0.062	0.250	0.125	0.500
	C	0.031	0.062	0.125	0.250
	T	0.031	0.125	0.062	0.250
74CCH	O	0.062	0.250	0.125	0.500
	C	0.015	0.031	0.062	0.125
	T	0.062	0.125	0.125	0.250
S. epidermidis					
ATCC 35984	O	0.125	0.500	0.250	1
	C	0.031	0.125	0.125	0.500
	T	0.062	0.125	0.125	0.500
14 ME	O	0.062	0.125	0.125	0.500
	C	0.031	0.125	0.125	0.500
	T	0.031	0.062	0.062	0.125
807 CT	O	0.125	0.500	0.250	1
	C	0.031	0.062	0.062	0.125
	T	0.031	0.125	0.062	0.125
813	O	0.125	0.250	0.250	0.500
	C	0.031	0.125	0.062	0.125
	T	0.031	0.062	0.062	0.125
23 S	O	0.125	0.250	0.250	0.250
	C	0.015	0.031	0.031	0.125
	T	0.031	0.031	0.062	0.125
809	O	0.125	0.250	0.250	0.500
	C	0.015	0.031	0.062	0.250
	T	0.031	0.062	0.062	0.125

Table 2. Effects of oregano (O), carvacrol (C) and thymol (T) on biofilm formation

Strain	Agent	Biofilm formation*		
		0.5 MIC	0.25 MIC	0.125 MIC
S. aureus				
6ME	O	52.45±6.3	73.3±8.2	103±10
	C	34.8±5.1	90.8±9.5	96.8±9.8
	T	49.9±3	98.4±9.6	96.9±7.2
810 CT	O	50.3±6.1	63.4±7.3	93.9±9
	C	41.2±5.6	75.6±8.4	76.5±8.4
	T	20.9±1.2	72.6±8	76.8±8.4
815 CT	O	10.6±1.4	17.1±4.1	79.33±8.6
	C	3.1±4.2	19.9±5	62±3.2
	T	3.48±3.2	20±2	71.3±8.4
808 CT	O	6.23±5.2	30.7±3.6	95.9±9.7
	C	8.3±2.5	25.9±2.7	35±7.4
	T	14.9±1.5	20±2	48±5.9
5 ME	O	50±1.2	78.1±2.3	95±6
	C	40.1±3	75.2±4	98.4±10.1
	T	49.1±2.3	72±1.3	96.9±2.3
74CCH	O	24.4±2.3	53.4±6.4	83±8.8
	C	32.3±4.6	66.4±7.8	101±8.2
	T	23.2±2	73.27±8.1	118±11.2
S. epidermidis				
ATCC 35984	O	65±4.9	72.4±8	98.8±9.6
	C	70.9±8.2	99±9.9	98.16±9.9
	T	71.67±8	102±10	102±10.1
14 ME	O	33.46±2	81.9±8.5	95.88±7.6
	C	40±3.2	64.6±6.8	104±10.3
	T	31.24±4	36±2	71.5±7.5
807 CT	O	58±6.9	85.6±9	103±10
	C	22.9±1.9	41.6±5.2	81.7±8.78
	T	22.56±1.8	28.5±3	64.1±7.4
813 CT	O	25.5±2.6	72.4±8.1	111±10.6
	C	48±3	79.2±8.6	87.5±9.2
	T	40.1±1.3	75.8±8.3	98.1±9.8
23 S	O	81.8±8	107.3±10	101±10
	C	47.2±5.8	93.9±9.6	106±10.4
	T	25.2±2.5	60.5±7	76.8±8.4
809	O	70±7.9	94.4±9.6	97.3±9.8
	C	43.4±5.4	65.3±7.5	86±4.1
	T	24±2.2	73.9±8.2	82.6±8.8

*Biofilm formation values were calculated as: (mean OD_{492} treated well)/(mean OD_{492} control well) × 100. Values are expressed as means ± SD.

2004), we show here that these oils inhibited growth of preformed biofilm and interfered with biofilm formation during planktonic growth. The reasons for this could be due to many factors acting either synergistically or alone. The antimicrobial activity of oregano oil is mostly attributed to the action of its principal phenolic components, carvacrol and thymol, which exhibit significant bactericidal activity when tested separately (Friedman *et al.*, 2002; Juven *et al.*, 1994; Lambert *et al.*, 2001; Ultee *et al.*, 1998). Due to their hydrophobic nature, carvacrol and thymol interact with the lipid bilayer of cytoplasmic membranes causing loss of integrity and leakage of cellular material such as ions, ATP and nucleic acid (Helander *et al.*, 1998; Lambert *et al.* 2001; Trombetta *et al.*, 2005; Ultee *et al.*, 1999). The extent of membrane damage induced by a compound can be related to its intrinsic hydrophobicity, which can be determined experimentally by its partition coefficient in octanol/water ($P_{o/w}$). Carvacrol and thymol have a log $P_{o/w}$ of 3.64 and 3.30, respectively (Griffin *et al.*, 1999; Ultee *et al.*, 2002). Weber & de Bont (1996) reported that compounds with a log $P_{o/w}$ value higher than 3 will partition deeply in the cell membrane. However, carvacrol and thymol have been reported to possess a relative

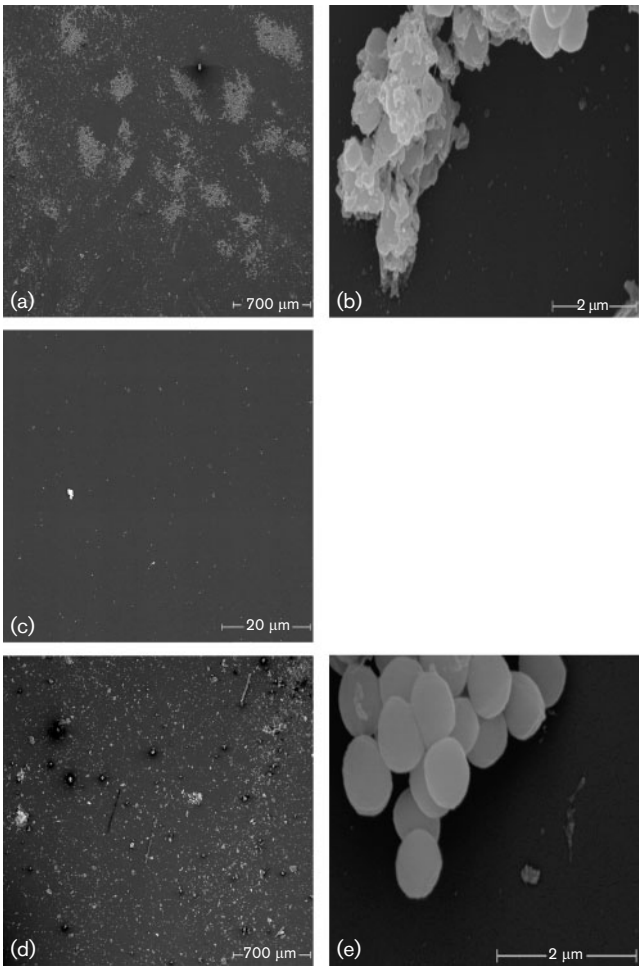

Fig. 1. Scanning electron micrographs of *S. aureus* 815 biofilms formed on polystyrene flat-bottomed microtitre plates: (a, b) biofilm control, (c) biofilm formed in the presence of 0.5 MIC of carvacrol, (d, e) biofilm formed in the presence of 0.25 MIC of carvacrol.

hydrophilicity, having a water solubility of 830 ± 10 and 846 ± 9 p.p.m., respectively (Griffin *et al.*, 1999). Hence, carvacrol and thymol, alone or in oregano oil, could diffuse through the polysaccharide matrix of the biofilm and destabilize it due to their strong intrinsic antimicrobial properties. 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).

Carvacrol and thymol may also be responsible for the effects observed on biofilm formation. Knowles *et al.* (2005) suggested that continued exposure of *S. aureus* to non-biocidal concentrations of carvacrol disrupted normal development of dual-species biofilm, preventing the build up of protein mass, arresting at the microcolony stage. Alternatively, the oils could interact with surface proteins, leading to an alteration of the bacterial cell surface and in part compromising the initial attachment phase to polystyrene microtitre plates. In this context, Juven *et al.* (1994) suggested a reaction between phenolic compounds (such as thymol) and bacterial membrane proteins.

The findings of the present study highlight the promising role of oregano, carvacrol and thymol as new lead structures in the search for novel antibacterial agents. Data in the literature on the availability and pharmacokinetics of carvacrol and thymol (Bhattaram *et al.*, 2002; De Vincenzi *et al.*, 2004), and on acute and short-term *in vivo* effects, suggest that they may not pose a risk for human and animal health (Chami *et al.*, 2005; Stammati *et al.*, 1999). Therefore, further experiment may be worthy of evaluation.

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