Assessment of the antibacterial activity of tea tree oil using the European EN 1276 and EN 12054 standard suspension tests

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Summary The activity of tea tree oil (TTO) and TTO-containing products was investigated according to the EN 1276 and EN 12054 European suspension methods. The activity of different concentrations of TTO, a hygienic skin wash (HSW), an alcoholic hygienic skin wash (AHSW) and an alcoholic hand rub (AHR) was investigated. These formulations were assessed in perfect conditions with the EN 12054 test, and in perfect conditions as well as in the presence of interfering substances with the EN 1276 test, against *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli* and *Pseudomonas aeruginosa*. With the latter test, the activity of the same formulations without TTO was also assessed as a control. With the EN 1276 test, the AHR achieved a $>10^5$-fold reduction against all four test organisms within a 1-min contact time. The AHSW achieved a $\geq 10^5$-fold reduction against *A. baumannii* after a 1-min contact time and against *S. aureus*, *E. coli* and *P. aeruginosa* after a 5-min contact time. The efficacy of TTO appeared to be dependent on the formulation and the concentration tested, the concentration of interfering substances and, lastly, the organism tested. Nevertheless, 5% TTO achieved a $>10^4$-fold reduction in *P. aeruginosa* cell numbers after a 5-min contact time in perfect conditions. TTO (5%) in 0.001% Tween 80 was significantly more active against *E. coli* and *P. aeruginosa* than against *S. aureus* and *A. baumannii*. With the EN 12054 test, after a 1-min contact time, 5% TTO in 0.001% Tween 80 and the AHSW achieved a $>10^4$-fold reduction in *E. coli* and *A. baumannii* cell numbers, respectively, and the AHR achieved a $>4 \log_{10}$ reduction

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against all organisms tested. The formulations used in this study are now being tested using a novel ex vivo method as well as the in vivo European standard handwashing method EN 1499.

Introduction

Morbidity and mortality due to life-threatening hospital-acquired infections remains a significant problem in health care today. The major mode of infection transmission is still thought to be hand carriage of pathogens from staff to patient, and from patient to patient.[1-3] Despite clear evidence that appropriate handwashing and skin antisepsis play a major role in reducing the spread of infections in hospital settings,[2,4,5] compliance with hand-hygiene practices is still unacceptably low.[3,6,7] Most soaps and detergents can be damaging to the skin when applied routinely during handwashing, leading to a change in microbial flora, an increase in bacterial shedding and thus an increased risk of transmission of micro-organisms.[1,8]

The essential oil of *Melaleuca alternifolia* [tea tree oil (TTO)] has been used medicinally for about 80 years.[9] TTO has broad-spectrum antimicrobial[9] and anti-inflammatory[10,11] activity in vitro. Hammer *et al.*[12] showed that transient skin organisms were more susceptible to TTO than commensal organisms. This finding supports the use of TTO-containing handwash products since the normal skin flora represents one of the natural defences against colonization by pathogenic organisms.[13] Other reports have suggested that the repeated use of TTO-containing hand wash does not lead to the dermatological problems associated with some formulations,[14] and this finding might be used to encourage healthcare staff's compliance with handwashing. Although the antibacterial activity of TTO has been well established in vitro,[11,15-19] TTO has not yet been assessed using European standard methods that are now widely accepted for the evaluation of disinfectant and antiseptic efficacy. In this study, we assessed the activity of TTO and TTO-containing formulations according to two European standard suspension methods, EN 1276[20] and prEN 12054.[21]

Materials and methods

Micro-organisms

Staphylococcus aureus (ATCC 25923), *Acinetobacter baumannii* (NCTC 7844), *Escherichia coli* K12 (NCTC 10538) and *Pseudomonas aeruginosa* (NCTC 6749) stock cultures were made on tryptone soya agar (TSA; Oxoid, Basingstoke, Hampshire, UK) plates, stored at 4 °C and renewed once a week.

Working cultures of bacteria

Subcultures on to further TSA plates were prepared from the stock cultures and incubated for 18–24 h at 37 °C. From this second subculture, a third subculture was produced in the same way. As recommended by European standard method EN 1276,[20] the second and/or third subcultures were the working culture(s).

To prepare bacterial test suspensions, loopfuls of cells from the working cultures were transferred into 15 mL of diluent, containing 1 g/L tryptone pancreatic digest of casein (Difco, Becton, Dickinson and Co., Sparks, MD, USA) and 8.5 g/L sodium chloride [BDH AnalaR, Merck Pty Ltd, Victoria, Australia, (BDH)] at pH 7, which was placed in a 100-mL flask with 5 g of glass beads. The flask was shaken for 3 min using a mechanical shaker (VorMix, Scientific Equipment Manufacturers, Brisbane, Australia). The suspension was then aspirated from the glass beads and transferred into another tube. Using a nephelometer, the number of cells in the suspension was adjusted with diluent to approximately $1.5-5 \times 10^8$ cfu/mL when performing the EN 1276 suspension test, and to approximately $1-3 \times 10^8$ cfu/mL when performing the EN 12054 suspension test. These suspensions were used within 2 h.

Antiseptic products

Suspension test EN 1276[20]

The antimicrobial activity of the following agents was investigated: an hygienic skin wash (HSW) containing 5% TTO (100% HSW; Novasel Australia Pty Ltd, Mudgeeraba Queensland, Australia); an alcoholic hygienic skin wash (AHSW) containing 5% TTO and 10% alcohol (100% AHSW; Novasel); an alcoholic hand rub (AHR) containing 3% TTO and 64% alcohol (100% AHR; Novasel); and 1, 2, 3, 4, 5, 6, 8 and 10% TTO in sterile distilled water (SDW) with 0.001% Tween 80 (Sigma Chemical Co., St Louis, MO, USA). TTO was kindly supplied by Australian Plantations Pty Ltd, Wyrallah, NSW, Australia. Batch W\EU04 complied

with the international standard for TTO (ISO 4730)[22] and contained 38.6% terpinen-4-ol and 3.6% 1,8 cineole. Levels of components were assessed by gas chromatography/mass spectrometry, performed by the Wollongbar Agricultural Institute, Wollongbar, NSW, Australia. Antimicrobial products were diluted in SDW to the concentration indicated and tested immediately.

For comparative purposes, in most experiments, the activity of a widely accepted antiseptic, 7.5% (v/v) povidone iodine (PVI; Orion Laboratories Pty Ltd, Welshpool, Western Australia), was tested. As the aim was to assess whether or not TTO was the main active component of some of the formulations tested, the efficacy of HSW without TTO (HSW-TTO), AHR without TTO (AHR-TTO) and 0.001% Tween 80 was assessed.

Suspension test prEN 12054[21]
As described in the standard method, the formulations under test, 100% HSW, 100% AHSW and, as controls, 5% TTO in 0.001% Tween 80 and 7.5% PVI were diluted to 55% (v/v) prior to testing. AHR (100%) was not diluted.

Neutralizer

The neutralizing solution used to quench the activity of antiseptics was based on European standard EN 1276[20] and contained: 30 g/L Tween 80 (Sigma), 3 g/L lecithin (Sigma), 1 g/L histidine (Sigma), 5 g/L sodium thiosulphate (BDH) and 34 g/L potassium dihydrogen phosphate (BDH) in tryptone soya broth (Oxoid). Two controls were required to validate this neutralizer: first, the neutralizer's toxicity had to be assessed against the test bacteria, and second, its ability to quench the corresponding antimicrobial activity had to be confirmed.

Antibacterial activity of the neutralizer
One millilitre of a working bacterial solution of *S. aureus*, *A. baumannii*, *E. coli* or *P. aeruginosa* cells was added to 8 mL of neutralizer and 1 mL of SDW. After 5 min of contact, this solution was serially diluted in sterile phosphate buffered saline (PBS) [8 g/L NaCl (BDH), 0.2 g/L KCl (BDH), 1.44 g/L Na_2HPO_4 (BDH) and 0.24 g/L KH_2PO_4 (BDH)]. The number of cfu/mL recovered was assessed by the drop counting method (DCM).[23]

Dilution-neutralization validation
The ability of the neutralizer to quench the corresponding antiseptic was assessed as follows: 1 mL of SDW was mixed with 1 mL diluent. Eight millilitres of antiseptic solution was added to this mixture and,

after 1 min, 1 mL was transferred into a test tube containing 8 mL of neutralizer. After 5 min, 1 mL of bacterial solution was added to the mixture and left in contact for 30 min. The final mixture was then serially diluted and counted using the DCM.

Interfering substances

As recommended by EN 1276, the interfering substance tested was a bovine albumin solution, under clean [0.3 g/L bovine albumin (Sigma)] or dirty (3 g/L bovine albumin) conditions. When the antiseptics were assessed in 'perfect' conditions, the interfering substance was replaced by SDW.

EN 1276 suspension test

This quantitative suspension test method was designed for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, domestic and industrial areas. The requirement of this standard is a minimum reduction by a factor of 10^5 within 5 min. The antiseptic formulations were also assessed after a 1-min contact time, to better reflect real-life conditions.

One millilitre of interfering substance (or SDW for perfect conditions) was mixed with 1 mL of bacterial test suspension. After 2 min, 8 mL of one of the product solutions was added to the mixture and shaken. After 1 and 5 min, 1 mL of the test mixture was transferred into a tube containing 8 mL of neutralizer and 1 mL of SDW, and mixed. After a neutralization time of 5 min, solutions were serially diluted and viable counts were performed on TSA plates using the DCM. The DCM was used as log_{10} reductions <4 cannot be measured with the counting method recommended by the European standard. Plates were incubated for 24 h at 37 °C, counted, and then re-incubated for a further 24 h to detect slow-growing colonies.

In this study, the aim was to compare the activity of: (i) 5% TTO in Tween 80 with that of 7.5% PVI, 100% HSW, 100% AHSW and 100% AHR in perfect, clean and dirty conditions; (ii) different concentrations of TTO in 0.001% Tween 80; (iii) 5% TTO in Tween 80 with that of 0.001% Tween 80 alone; (iv) 100% HSW with that of 100% HSW without TTO; and (v) 100% AHR with that of 100% AHR without TTO.

prEN 12054 suspension test

This quantitative suspension test method was designed for testing hygienic and surgical handrub and handwash products. The requirements of this standard are a minimum log_{10} reduction factor of

Table I Effect of the neutralizer (EN 1276) on the number of bacterial cells after a 5-min challenge

Bacteria	Log_{10} bacterial cell numbers recovered after treatment, mean (SD)
Staphylococcus aureus	-0.003 (0.072)
Acinetobacter baumannii	$+0.216$ (0.137)
Escherichia coli	$+0.270$ (0.119)
Pseudomonas aeruginosa	$+0.065$ (0.143)

2.52 in cfu/mL for a hygienic hand wash and $\log_{10}$ of 4.52 for an hygienic hand rub within 1 min.

One millilitre of bacterial test suspension was mixed with 9 mL of 55% HSW, 55% AHSW, 2.75% TTO in Tween 80 or 100% AHR in a sterile McCartney bottle. After 1 and 5 min, 1 mL of this mixture was transferred into a bottle containing 8 mL of neutralizer and 1 mL of SDW. After a neutralization time of 1 min, the solutions were serially diluted in PBS and viable counts were performed on TSA using the DCM. The appropriate contact time for hygienic hand washes and hand rubs should be 1 min. However, in order to assess whether or not a longer contact time would significantly influence the activity of the formulations, a 5-min contact time was also used. All experiments were conducted at room temperature: $20\,^{\circ}\text{C} \pm 1\,^{\circ}\text{C}$.

Statistical analysis

Analyses of variance were performed with the Excel® (Excel, Microsoft Corporation, Redmond, WA, USA) and Prism® (Graphpad Prism, Graphpad Software Inc., San Diego, CA, USA) software packages. Three to five replicates were performed and *P* values of <0.05 were considered to be significant.

Results

Antibacterial activity and efficacy of the neutralizer

As shown in Table I, the neutralizer did not reduce

the concentration of bacterial cells significantly ($P > 0.05$). The neutralizing solutions quenched the antiseptics effectively ($P > 0.05$) at the concentrations tested (Table II).

EN 1276 suspension test

Antibacterial activity of the TTO-containing antiseptics

According to the European standard method used, the products under test had to achieve $\log_{10}$ reduction in bacterial cell numbers of at least after a 1- or 5-min contact time. Products that complied with the European standard are listed in Table III. After a 1-min contact time, the only products that achieved a $\log_{10}$ reduction of 5 against all four bacteria were 100% AHR (in perfect and clean conditions) and 7.5% PVI (in perfect, clean and dirty conditions). After a 5-min contact time, the products that achieved $\log_{10}$ reduction of 5 against all four bacteria were the AHR (in perfect and clean conditions), the AHSW (in perfect and clean conditions) and PVI (in perfect, clean and dirty conditions).

It is interesting to note that different concentrations of TTO in Tween 80 (from 4 to 10%) also achieved a $\log_{10}$ reduction of 5, but only against *E. coli* and *P. aeruginosa*. In addition, *A. baumannii* and *E. coli* were susceptible to a wider range of products and lower concentrations than *S. aureus* and *P. aeruginosa* (HSW and AHR concentrations ranging from 10 to 75% were also assessed, data not shown).

TTO in 0.001% Tween 80.
When assessed against *S. aureus*, 5% TTO in 0.001%

Table II Neutralizer's efficacy in quenching the activity of different antiseptics after a 5-min challenge

Bacterial strains	Log_{10} bacterial cell numbers recovered after treatment, mean (SD)		
	5% TTO	7.5% PVI	100% AHR
		+ Neutralizer (EN 1276)	
Staphylococcus aureus	-0.187 (0.162)	$+0.024$ (0.062)	-0.212 (0.152)
Acinetobacter baumannii	$+0.167$ (0.158)	$+0.142$ (0.131)	$+0.273$ (0.149)
Escherichia coli	$+0.077$ (0.020)	$+0.089$ (0.101)	-0.251 (0.077)
Pseudomonas aeruginosa	-0.271 (0.220)	$+0.283$ (0.105)	$+0.0895$ (0.187)

TTO, tea tree oil; PVI, povidone iodine (control antiseptic); AHR, alcoholic hand rub.

Table III   List of products that achieved at least a 5 $\log_{10}$ reduction for various contact times and bacteria tested

Bacteria	Time	
	1 min	5 min
Staphylococcus aureus		100% AHSW (P/C/D)
	100% AHR (P/C/D)	100% AHR (P/C/D)
	7.5% PVI (P/C/D)	7.5% PVI (P/C/D)
Acinetobacter baumannii	100% HSW (C)	100% HSW (P)
	100% AHSW (P/C/D)	100% AHSW (P/C/D)
	100% AHR (P/C/D)	100% AHR (P/C/D)
	7.5% PVI (P/C/D)	7.5% PVI (P/C/D)
Escherichia coli		1% TTO (P)
		4% TTO (P)
	5% TTO (P/C/D)	5% TTO (P/C/D)
	6% TTO (P)	6% TTO (P)
	8% TTO (P)	8% TTO (P)
	10% TTO (P)	10% TTO (P)
		100% HSW (P)
	100% AHSW (C)	100% AHSW (P/C)
	100% AHR (P/C)	100% AHR (P/C)
	7.5% PVI (P/C/D)	7.5% PVI (P/C/D)
Pseudomonas aeruginosa		4% TTO (P)
	6% TTO (P)	
	8% TTO (P)	8% TTO (P)
	10% TTO (P)	10% TTO (P)
		100% AHSW (P/C/D)
	100% AHR (P/C)	100% AHR (P/C)
	7.5% PVI (P/C/D)	7.5% PVI (P/C/D)

P, perfect conditions; C, clean conditions; D, dirty conditions; TTO, tea tree oil; PVI, povidone iodine (control antiseptic); AHR, alcoholic hand rub; HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash.

Tween 80 was more active in perfect conditions although the difference was not significant ($P > 0.05$). Also, its activity generally increased after a 5-min contact time, but this was only significant ($P < 0.05$) when assessed in perfect conditions (Figure 1). When tested against *A. baumannii*, there was no significant ($P > 0.05$) difference in activity, regardless of the tests being performed in perfect or clean conditions, after 1- and 5-min contact times. However, TTO was more active when tested in perfect conditions than in dirty conditions, and this difference was significant ($P < 0.05$) after a 5-min contact time (Figure 2). Regardless of the conditions, TTO was always significantly more active after a 5-min contact time (Figure 2). When assessed against *E. coli*, TTO was equally active ($\log_{10}$ reduction ≥ 5) regardless of whether the tests were performed in perfect, clean or dirty conditions after 1- and 5-min contact times (Figure 3). Against *P. aeruginosa*, TTO was more active when assessed in perfect conditions than in clean conditions, but this was only significant ($P < 0.05$) after a 5-min contact time. TTO was significantly ($P < 0.05$) more active in perfect than dirty conditions (Figure 4). TTO was significantly

more active ($P < 0.05$) after a 5-min contact time compared with a 1-min contact time when assessed in perfect conditions. However, there was no significant difference ($P > 0.05$) in the activity of TTO depending upon the contact time when assessed in clean and dirty conditions (Figure 4).

7.5% PVI.
The different conditions (perfect, clean or dirty) and contact times did not significantly affect the antibacterial activity ($P > 0.05$) of PVI (Figures 1–4). PVI (7.5%) was significantly ($P < 0.05$) more active than 5% TTO against *S. aureus*, *A. baumannii* and *P. aeruginosa* (Figures 1, 2 and 4), while TTO was as active ($P > 0.05$) as PVI against *E. coli* (Figure 3).

HSW.
When assessed against *S. aureus*, 100% HSW was generally more active ($P < 0.05$) in dirty than clean conditions, and after a 5-min contact time. However, this was only significant ($P < 0.05$) when assessed in dirty conditions (Figure 1). Against *A. baumannii*, conditions did not significantly influence the activity of 100% HSW after a 1-min contact time. HSW (100%) was
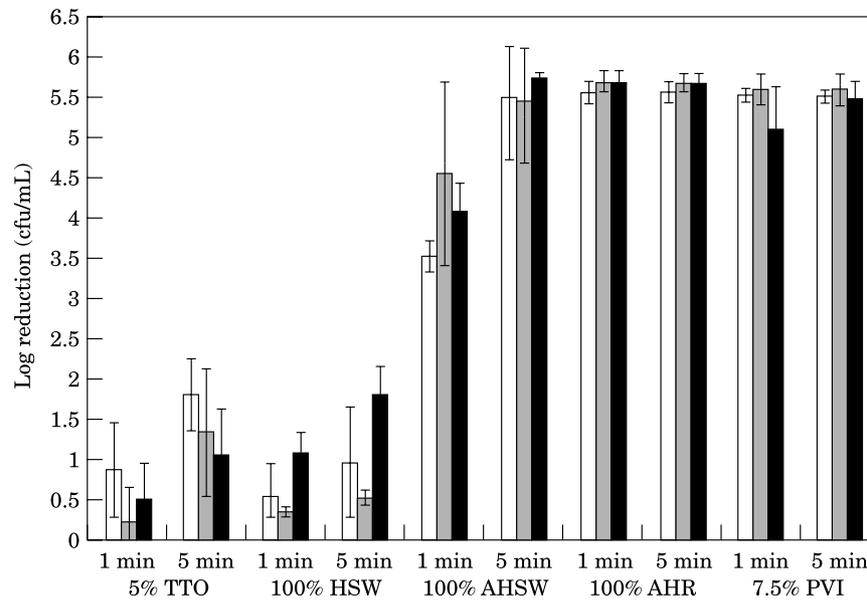
Figure 1 EN 1276. Antibacterial activity of 5% tea tree oil (TTO), TTO-containing products and 7.5% povidone iodine (PVI, control antiseptic) against *Staphylococcus aureus* in different test conditions: perfect (open bars), clean (stippled bars) or dirty (solid bars) conditions (mean ± SD). HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash; AHR, alcoholic hand rub.

more active in perfect and clean conditions after a 5-min contact time, but this was not significant ($P > 0.05$) (Figure 2). There were no significant ($P > 0.05$) differences when 100% HSW was assessed in perfect, clean or dirty conditions after a 1-min contact time against *E. coli*. However, after a 5-min contact time, 100% HSW was less active ($P < 0.05$) in dirty than

perfect conditions (Figure 3). HSW was significantly ($P < 0.05$) more active after a 5-min contact time than a 1-min contact time (Figure 3). Surprisingly, when tested against *P. aeruginosa*, 100% HSW was significantly more active ($P < 0.05$) when assessed in both clean and dirty rather than perfect conditions after a 1-min contact time. However, there was no significant
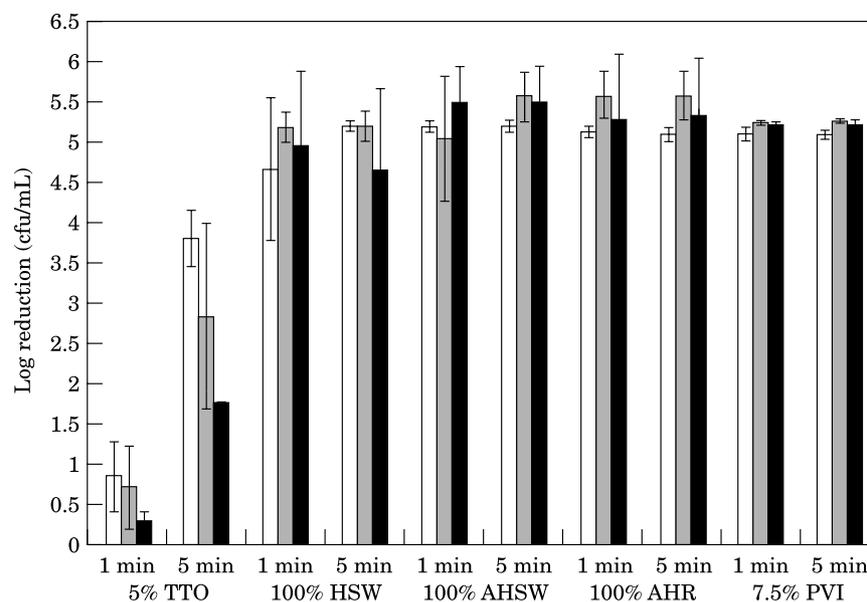

Figure 2 EN 1276. Antibacterial activity of 5% tea tree oil (TTO), TTO-containing products and 7.5% povidone iodine (PVI, control antiseptic) against *Acinetobacter baumannii* in different test conditions: perfect (open bars), clean (stippled bars) or dirty (solid bars) conditions (mean ± SD). HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash; AHR, alcoholic hand rub.
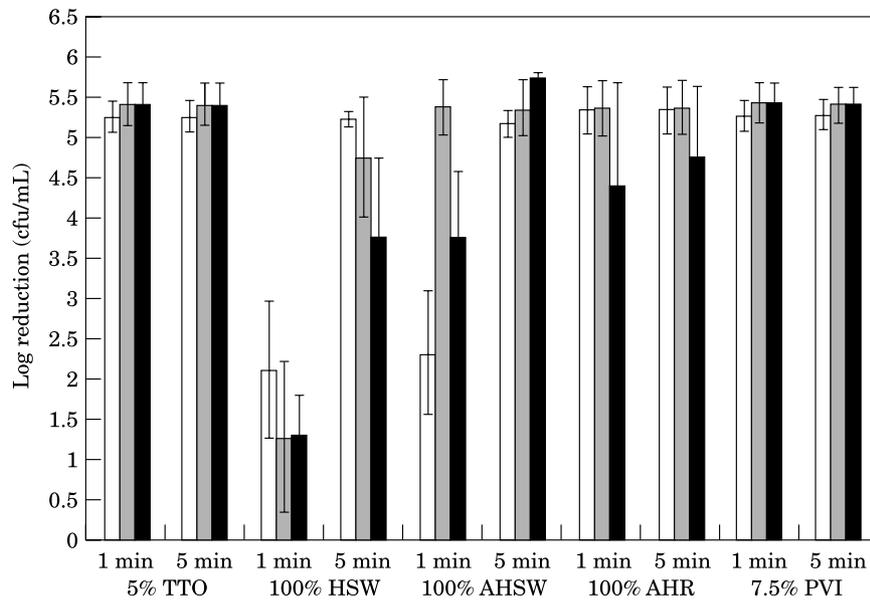
Figure 3 EN 1276. Antibacterial activity of 5% tea tree oil (TTO), TTO-containing products and 7.5% povidone iodine (PVI, control antiseptic) against *Escherichia coli* in different test conditions: perfect (open bars), clean (stippled bars) or dirty (solid bars) conditions (mean ± SD). HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash; AHR, alcoholic hand rub.

difference in activity between conditions after a 5-min contact time (Figure 4).

TTO (5%) was more active than 100% HSW against *S. aureus* in perfect conditions after 1- and 5-min contact times, but this difference was not significant ($P > 0.05$) (Figure 1). After a 1-min contact time, 5% TTO was always significantly ($P < 0.05$) more active than 100% HSW against *E. coli*, but after

a 5-min contact time, there was no significant ($P > 0.05$) difference in activity between the two products (Figure 3). In perfect conditions, 5% TTO was significantly ($P < 0.05$) more active than HSW against *P. aeruginosa* after 1- and 5-min contact times. However, in clean and dirty conditions, 100% HSW was significantly ($P < 0.05$) more active than 5% TTO after a 5-min contact time (Figure 4). Finally,
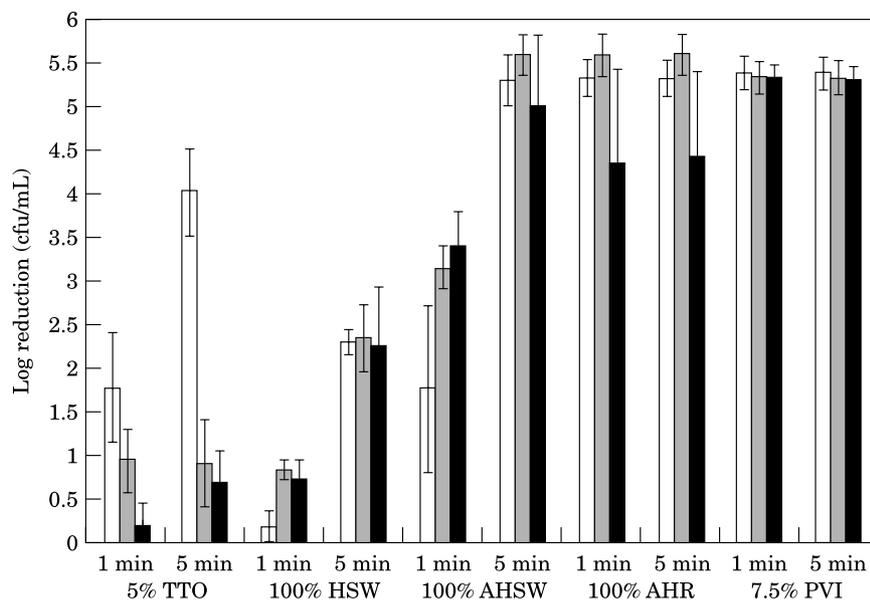

Figure 4 EN 1276. Antibacterial activity of 5% tea tree oil (TTO), TTO-containing products and 7.5% povidone iodine (PVI, control antiseptic) against *Pseudomonas aeruginosa* in different test conditions: perfect (open bars), clean (stippled bars) or dirty (solid bars) conditions (mean ± SD). HSW, hygienic skin wash; AHSW, alcoholic hygienic skin wash; AHR, alcoholic hand rub.

5% TTO was significantly ($P<0.05$) less active than 100% HSW against *A. baumannii* after 1- and 5-min contact times (Figure 2).

AHSW.
When assessed against *S. aureus*, the different conditions (perfect, clean or dirty) did not affect the efficacy of 100% AHSW and its activity was increased after a 5-min contact time although this was only significant ($P<0.05$) in perfect and dirty conditions (Figure 1). There was no significant influence of the conditions (perfect, clean or dirty) and/or the contact time on the activity of 100% AHSW against *A. baumannii* (Figure 2). After a 1-min contact time, 100% AHSW was surprisingly more active ($P<0.05$) when assessed in clean or dirty conditions than in perfect conditions against *E. coli* and *P. aeruginosa* (Figures 3 and 4). Nevertheless, after a 5-min contact time, 100% AHSW was more active when assessed in perfect and clean conditions, although this was not significant ($P>0.05$) (Figures 3 and 4).

AHSW (100%) was significantly more active ($P<0.05$) than 5% TTO, regardless of the contact time and/or the conditions, against *S. aureus* and *A. baumannii* (Figures 1 and 2). After a 1-min contact time, 5% TTO was significantly ($P<0.05$) more active than 100% AHSW in perfect and dirty conditions against *E. coli*. However, in clean conditions, there was no significant difference in activity ($P>0.05$) between the two products (Figure 3). After a 5-min contact time, there was no significant difference in activity ($P>0.05$) between 5% TTO and 100% AHSW regardless of the conditions (Figure 3). When assessed against *P.*

aeruginosa, 100% AHSW was significantly more active ($P<0.05$) than 5% TTO in clean and dirty conditions after a 1-min contact time, and significantly more active ($P<0.05$) than 5% TTO regardless of the conditions after a 5-min contact time (Figure 4).

AHR.
The 100% AHR had good activity ($\log_{10}$ reduction >5) against *S. aureus*, *A. baumannii*, *E. coli* and *P. aeruginosa* regardless of the conditions and/or contact time ($P>0.05$) (Figures 1–4). The activity was slightly affected when assessed in dirty conditions against *E. coli* and *P. aeruginosa*, although this was not significant ($P>0.05$) (Figures 3 and 4). AHR was significantly ($P<0.05$) more active than 5% TTO at both contact times and regardless of the test conditions against *S. aureus*, *A. baumannii* and *P. aeruginosa* (Figures 1, 2 and 4). There was no significant ($P>0.05$) difference in activity between 5% TTO and 100% AHR when tested against *E. coli* (Figure 3).

Antibacterial activity of different concentrations of TTO
The different concentrations of TTO did not differ significantly ($P>0.05$; ANOVA) in their activity against *S. aureus*, regardless of the contact time (Table IV). Activity was always increased after a 5-min contact time, but this was only significant ($P<0.05$) for 2, 5 and 10% (Table IV). After a 1-min contact time, 1, 2 and 4% TTO had a comparable activity ($P>0.05$; ANOVA) against *A. baumannii*, although, after a 5-min contact time, 1% TTO was significantly less active ($P<0.05$) than 2% TTO, and

Table IV Summary of suspension test (EN 1276) results under perfect conditions

Antibacterial agent	Contact time	*Staphylococcus aureus*	*Acinetobacter baumannii*	*Escherichia coli*	*Pseudomonas aeruginosa*
1% TTO	1 min	0.19 (0.36)[a]	0.13 (0.39)	4.38 (1.25)	0.22 (0.25)
	5 min	0.92 (0.63)	0.85 (0.41)	5.26 (0.11)	0.93 (0.58)
2% TTO	1 min	0.40 (0.43)	−0.01 (0.06)	4.60 (0.93)	0.64 (0.21)
	5 min	1.39 (0.64)	1.83 (0.39)	4.84 (0.93)	2.24 (0.60)
4% TTO	1 min	0.61 (0.53)	0.53 (0.41)	4.77 (1.05)	2.05 (0.48)
	5 min	1.24 (0.74)	2.44 (0.52)	5.26 (1.20)	5.02 (0.91)
5% TTO	1 min	0.88 (0.58)	0.87 (0.43)	5.26 (1.20)	1.76 (0.62)
	5 min	1.80 (0.45)	3.81 (0.34)	5.26 (1.20)	4.02 (0.51)
6% TTO	1 min	0.26 (0.41)	1.11 (1.08)	5.26 (1.20)	5.12 (0.34)
	5 min	0.95 (0.71)	3.75 (1.43)	5.26 (1.20)	4.46 (0.57)
8% TTO	1 min	0.32 (0.10)	1.68 (0.94)	5.26 (1.20)	5.12 (0.34)
	5 min	1.16 (1.03)	4.72 (0.89)	5.26 (1.20)	5.29 (0.19)
10% TTO	1 min	0.80 (0.38)	3.04 (1.12)	5.26 (1.20)	5.12 (0.34)
	5 min	1.89 (0.60)	4.84 (0.68)	5.26 (1.20)	5.29 (0.19)

TTO, tea tree oil.
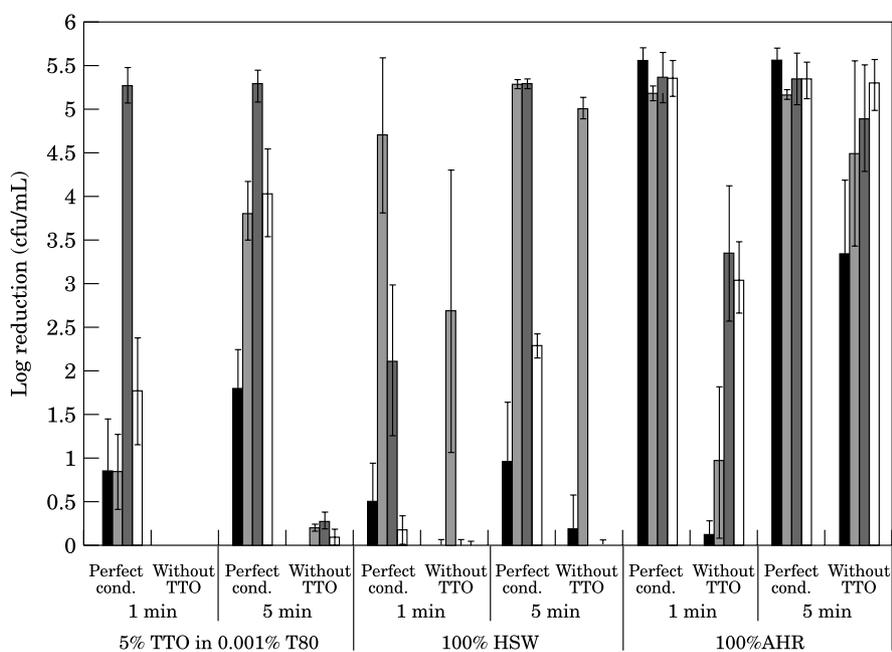[a] $\log_{10}$ reductions $\pm$ SD.

Figure 5 Antibacterial activity of 5% tea tree oil (TTO) in 0.001% Tween 80, 100% hygienic skin wash (HSW) and 100% alcoholic hand rub (AHR) (perfect conditions) compared with 0.001% Tween 80, 100% HSW-TTO and 100% AHR-TTO (mean±SD). Note: 0.001% Tween 80 was not tested at 1-min contact time. Solid bars, *Staphylococcus aureus*; striped bars, *Acinetobacter baumannii*; stippled bars, *Escherichia coli*; open bars, *Pseudomonas aeruginosa*.

2% TTO had a comparable activity to 4% TTO (Table IV). There was no significant difference in activity between 6, 8 and 10% TTO ($P > 0.05$; ANOVA), but 5% TTO was significantly less active than 10% TTO ($P < 0.05$, t-test) against *A. baumannii*. TTO was generally more active after a 5-min contact time than after a 1-min contact time, but this was only significant ($P < 0.05$) at 1, 2, 4, 5 and 8% (Table IV).

The different concentration and/or contact time did not affect the activity of TTO against *E. coli* significantly ($P > 0.05$; ANOVA) (Table IV). The activity of TTO against *P. aeruginosa* generally increased significantly ($P < 0.05$) with increasing concentration, although 6, 8 and 10% TTO had comparable activity ($P > 0.05$; ANOVA) after a 1-min contact time (Table IV). After a 5-min contact time, 5, 6, 8 and 10% TTO had comparable activity ($P > 0.05$; ANOVA). At all concentrations tested, TTO was generally more active after a 5-min contact time, but this was only significant ($P < 0.05$) at 1, 2, 4 and 5% (Table IV).

Antibacterial activity of formulations with or without TTO

The 0.001% Tween 80 solution did not have any significant ($P > 0.05$) antibacterial activity against any of the bacteria tested (Figure 5).

Hygienic skin wash.
When assessed against *S. aureus*, 100% HSW was

more active than 100% HSW-TTO after 1- and 5-min contact times, but this was not statistically significant ($P > 0.05$) (Figure 5). The 100% HSW was more active than 100% HSW-TTO against *A. baumannii*, but this was only significant ($P < 0.05$) after a 5-min contact time (Figure 5). When assessed against *E. coli* and *P. aeruginosa*, 100% HSW was significantly ($P < 0.05$) more active than 100% HSW-TTO after 1- and 5-min contact times (Figure 5).

AHR.
The 100% AHR was always significantly ($P < 0.05$) more active than 100% AHR-TTO against *S. aureus* (Figure 5). When assessed against *A. baumannii*, *E. coli* and *P. aeruginosa*, 100% AHR was only significantly ($P < 0.05$) more active than 100% AHR-TTO after a 1-min contact time (Figure 5).

Comparison between bacteria

TTO (5%) was significantly ($P < 0.05$) more active against *E. coli* than against *S. aureus*, *A. baumannii* and *P. aeruginosa* after 1- and 5-min contact times. After a 5-min contact time, 5% TTO was significantly more active ($P < 0.05$) against *A. baumannii* and *P. aeruginosa* than against *S. aureus*. *A. baumannii* was significantly ($P < 0.05$) more susceptible to 100% HSW than *E. coli*, which was significantly ($P < 0.05$) more susceptible than *S. aureus* and *P. aeruginosa* after a 1-min contact time. After a
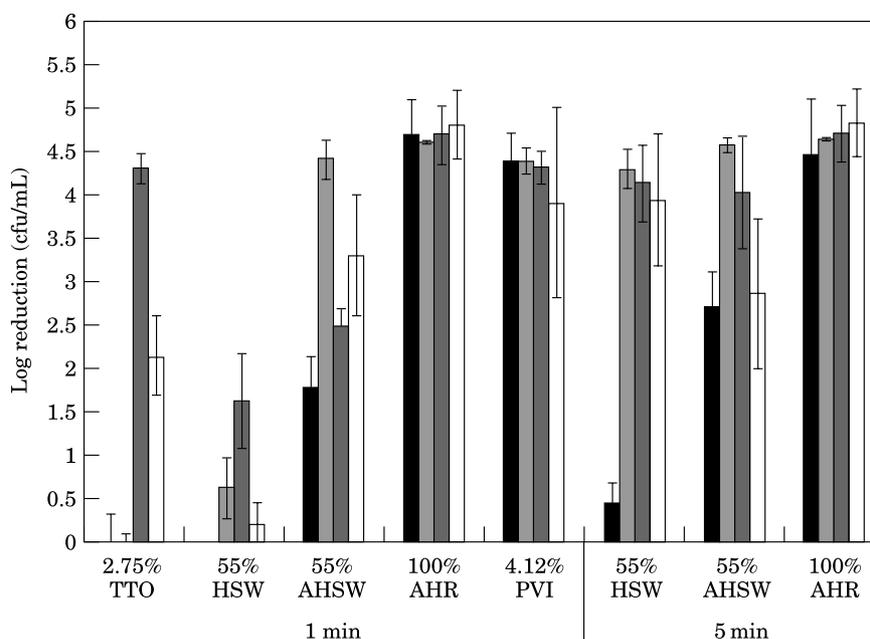
Figure 6 EN 12054. Antibacterial activity of tea tree oil (TTO), povidone iodine (PVI, control antiseptic), hygienic skin wash (HSW), alcoholic hygienic skin wash (AHSW) and alcoholic hand rub (AHR) against *Staphylococcus aureus* (solid bars), *Acinetobacter baumannii* (striped bars), *Escherichia coli* (stippled bars) and *Pseudomonas aeruginosa* (open bars) after 1-min and 5-min contact times (mean ± SD).

5-min contact time, *E. coli* and *A. baumannii* were both significantly ($P<0.05$) more susceptible to 100% HSW than *S. aureus* and *P. aeruginosa*.

A. baumannii was significantly ($P<0.05$) more susceptible to 100% AHSW than *S. aureus*, which was significantly ($P<0.05$) more susceptible than *E. coli* and *P. aeruginosa* after a 1-min contact time. All four bacteria were susceptible to 100% AHSW after a 5-min contact time, and to 100% AHR and 7.5% PVI after 1- and 5-min contact times. *E. coli* and *P. aeruginosa* were generally more susceptible than *S. aureus* and *A. baumannii* to different concentrations of TTO in Tween 80, although this was not always significant.

prEN 12054 suspension test

To comply with this standard method, a hygienic hand wash and a hand rub must achieve at least 2.523 $\log_{10}$ and 4.523 $\log_{10}$ reduction in bacterial numbers of $\log_{10}$, respectively, after the chosen contact time. The 100% AHR tested in this study achieved $\log_{10}$ reduction >4.52 in all bacteria within a 1-min contact time (Figure 6). The AHSW tested at 55% achieved a $\log_{10}$ reduction ≥ 2.52 against *A. baumannii*, *E. coli* and *P. aeruginosa*, while 4.12% PVI also complied with the standard method by achieving a $\log_{10}$ reduction ≥ 3.5 against all bacteria (Figure 6). After a 1-min contact time, 55% AHSW was always significantly more active than

the HSW regardless of the organism tested. *S. aureus* was always significantly less susceptible than the other bacteria to the formulations tested. The activity of HSW was generally increased with a longer contact time, although this was not always significant ($P>0.05$).

Discussion

Handwashing is one of the most important measures to reduce the transmission of infection in hospital settings.[24] However, compliance with hand-hygiene guidelines is low[6,7] and this can be partly attributed to the fact that most recommended handwash agents available in healthcare settings are irritant to the skin when used repetitively.[25] The efficacy of TTO has been demonstrated in vitro and its incorporation into skin products does not appear to cause the dermatological problems commonly reported with other formulations.[14] In this study, we used in-vitro methods based on European standards so that our results could be compared with other data in the literature as well as future work.

When assessed with suspension test EN 1276, 100% AHSW and 100% AHR, as well as 7.5% PVI, achieved a $\log_{10}$ reduction ≥ 5 after a 5-min contact time. Their activity was generally not influenced by the presence of interfering substances in suspension. Results obtained with the control antiseptic

(PVI) matched those obtained by Hill and Casewell[26] who reported that 5% PVI induced a $\log_{10}$ reduction of 5 in *S. aureus* cell numbers after a 1-min contact time, and those obtained by Maillard *et al.*[23] who showed a $\log_{10}$ reduction >5 in *E. coli* and *P. aeruginosa* cell numbers after a 1-min challenge with 2% PVI. Economou-Stamatelopoulou and Papavassiliou[27] also showed that the activity of 5% PVI was not significantly affected by the addition of interfering substances in suspension when assessed against *S. aureus*. In-vivo studies have shown 10% PVI to be the most effective agent, with 70% ethyl alcohol, to remove *A. baumannii*[28] and *S. aureus*[29] from the contaminated hands of volunteers. PVI is generally recommended for use in hospital settings as a surgical-scrub agent (7.5%)[30] and a hand-cleansing agent (10%).[28,29] However, PVI can irritate the skin[31] and its acceptance amongst healthcare staff varies. With EN 1276, the AHSW (after a 5-min contact time) and the AHR (after a 1-min contact time) were as effective as 7.5% PVI against all bacteria. Some reports suggest that the repeated use of a TTO-containing handwash does not lead to the dermatological problems associated with other formulations,[14] and this finding might be used to encourage healthcare staff's compliance with handwashing. In addition, Hammer *et al.*[12] showed that transient skin organisms were more susceptible to TTO than commensal organisms. Hence the formulations assessed in this study could prove useful in healthcare settings where removal of potentially pathogenic organisms is as important as preserving skin integrity and resident flora.

The activity of 5% TTO could generally be ranked according to the conditions in which it was tested: perfect $\geq$ clean $\geq$ dirty. However, its efficacy was only significantly affected when assessed against *P. aeruginosa* and *A. baumannii*. Walsh *et al.*[32] assessed the activity of the essential oils thymol and eugenol using European suspension test EN 1276. The activity of 0.1% (v/v) eugenol and 1000 μg/mL thymol against *E. coli* was reduced in dirty conditions, but the efficacy of thymol against *S. aureus* was not. As shown in the present study, the activity of essential oils appears to be affected by the conditions in which they are tested, and depends on the organisms assessed. It was interesting to find that 5% TTO in Tween 80 achieved $>4 \log_{10}$ reduction in *P. aeruginosa* cell numbers after a 5-min contact time, and 100% AHSW and 100% AHR achieved $>5 \log_{10}$ reduction in perfect and clean conditions after 5- and 1-min contact times, respectively. In comparison, thymol and eugenol did not achieve a $\log_{10}$ reduction in *P. aeruginosa* cells greater than 4.[32] The activity of

100% AHSW and 100% AHR against *E. coli* and *P. aeruginosa* was reduced in dirty conditions, although this reduction was not significant. Hammer *et al.*[33] demonstrated that no one single interfering substance affected the activity of TTO against the micro-organisms they tested. Our results, showing a different interference level depending upon the formulation and concentration of TTO tested, the concentration of interfering substances and the organism tested, agree with those of Hammer *et al.*[33]

It is as important to assess the activity of an active compound as it is to assess the final formulation, as synergy or antagonism can occur between the components of the essential oils and the ingredients of the formulation.[34] When the formulations that did not contain TTO were assessed, they were generally less active than those containing TTO. Even though it contained 64% alcohol, the AHR was significantly more active than its equivalent without TTO after a 1-min contact time against all bacteria, reinforcing the fact that the activity of this formulation is mainly due to the presence of TTO. The AHR passed the standard guidelines even though the concentration of TTO was only 2% in this formulation. The presence of a high concentration of alcohol probably enhanced the activity of the AHR. This was also supported by our results with the AHSW that achieved higher reductions in bacterial numbers than the HSW in both suspension tests, although they both contained 5% TTO. Synergism between alcohol and some other active compounds has been reported with chlorhexidine,[35,36] Triclosan[37] and PVI.[38]

Furthermore, the TTO-containing formulations assessed in this study also performed well according to the draft European standard method prEN 12054[21] for the assessment of hygienic handrub and handwash solutions. The AHSW successfully passed the European recommendations after a 1-min contact time against *E. coli*, *A. baumannii* and *P. aeruginosa*. The AHR also matched the European recommendations when tested against all bacteria. Previous studies have generally shown that AHRs have greater and faster activity than handwash products,[39,40] and are more 'skin-friendly' and hence more tolerated by healthcare staff.[41,42] The AHR tested in this study passed both European standard methods and the presence of TTO enhanced its activity. Alcohol-based formulations have recently been promoted as being the most important development for improving compliance with hand cleansing in understaffed and overcrowded situations.[2,25] In addition, they have been reported to cause less irritation and

skin dryness than commonly used soaps and anti-septics.[41,43,44] Thus, our findings regarding the TTO-containing AHR are very encouraging and should be investigated further. The activity of the HSW was significantly improved after a 5-min contact time against *E. coli*, *A. baumannii* and *P. aeruginosa*. However, in real-life conditions, healthcare staff are unlikely to wash their hands for longer than 1 min.

Previous studies have underlined the fact that suspension tests might overestimate the activity of disinfectants and antiseptics,[23,37] and thus active compounds should be assessed using practical tests. Suspension tests are indeed less severe than capacity tests and practical tests.[45] The efficacy of handwash formulations might be reduced in real-life conditions because of the presence of organic load, sweat and sebum on the skin. Nonetheless, the AHSW and AHR assessed in this study were shown to comply with the European standard against all bacteria tested. In addition, concentrations of TTO $\geq 5\%$ also complied with EN 1276 by achieving $\log_{10}$ reduction ≥ 5 when tested against *E. coli* and *P. aeruginosa*, and 100% HSW also complied when assessed in perfect conditions against *E. coli* and *A. baumannii*. This indicates that these products have at least met a minimum standard, and it remains for future investigations to evaluate them further.

In conclusion, the formulations tested in this study generally achieved high reduction in bacterial cell numbers. Their efficacy could be ranked as follows: 100% AHR $>$ 100% AHSW $>$ 5% TTO in Tween 80 $>$ 100% HSW. Both the TTO-containing AHSW and AHR might prove useful in hospital settings because of their efficacy in different testing conditions and because of the increasing acceptability of alcohol-based and TTO formulations as being less damaging to the skin. The efficacy of these formulations, as well as different concentrations of TTO in Tween 80, is currently being investigated by an ex vivo test that allows the testing of antiseptics directly on human skin.[23,37] The hygienic hand washes are also being assessed in a handwashing study based on European standard EN 1499.[46] Further studies could also include the testing of the TTO-containing AHR using European standard EN 1500[47] for the evaluation of hygienic hand rubs.

Acknowledgements

References

1. Larson E. Skin hygiene and infection prevention: more of the same or different approaches? *Clin Infect Dis* 1999;**29**: 1287–1294.
2. Boyce JM, Pittet D. Guideline for hand hygiene in health-care settings: recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. *Infect Control Hosp Epidemiol* 2002;**23**(Suppl. 12):S3–S40.
3. Naikoba S, Hayward A. The effectiveness of interventions aimed at increasing handwashing in healthcare workers—a systematic review. *J Hosp Infect* 2001;**47**:173–180.
4. Reybrouck G. Handwashing and hand disinfection. *J Hosp Infect* 1986;**8**:5–23.
5. Larson EL, Early E, Cloonan P, Sugrue S, Parides M. An organizational climate intervention associated with increased handwashing and decreased nosocomial infections. *Behav Med* 2000;**26**:14–22.
6. Pittet D. Compliance with hand disinfection and its impact on hospital-acquired infections. *J Hosp Infect* 2001; **48**(Suppl. A):S40–S46.
7. Karabey S, Ay P, Derbentli S, Nakipoglu Y, Esen F. Handwashing frequencies in an intensive care unit. *J Hosp Infect* 2002; **50**:36–41.
8. Larson E. Hygiene of the skin: when is clean too clean? *Emerg Infect Dis* 2001;**7**:225–230.
9. Carson CF, Riley TV. Antimicrobial activity of the essential oil of *Melaleuca alternifolia*. *Lett Appl Microbiol* 1993;**16**: 49–55.
10. Brand C, Ferrante A, Prager RH, *et al*. The water-soluble components of the essential oil of *Melaleuca alternifolia* (tea tree oil) suppress the production of superoxide by human monocytes, but not neutrophils, activated in vitro. *Inflamm Res* 2001;**50**:213–219.
11. Koh KJ, Pearce AL, Marshman G, Finlay-Jones JJ, Hart PH. Tea tree oil reduces histamine-induced skin inflammation. *Br J Dermatol* 2002;**147**:1212–1217.
12. Hammer KA, Carson CF, Riley TV. Susceptibility of transient and commensal skin flora to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Am J Infect Control* 1996;**24**: 186–189.
13. Noble WC. Skin bacteriology and the role of *Staphylococcus aureus* in infection. *Br J Dermatol* 1998;**139**(Suppl. 53): 9–12.
14. Carson CF, Riley TV. Toxicity of the essential oil of *Melaleuca alternifolia* or tea tree oil. *J Toxicol Clin Toxicol* 1995;**33**: 193–195.
15. Schnitzler P, Schon K, Reichling J. Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture. *Pharmazie* 2001;**56**:343–347.
16. Carson CF, Mee BJ, Riley TV. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. *Antimicrob Agents Chemother* 2002;**46**:1914–1920.
17. Hammer KA, Carson CF, Riley TV. In vitro activity of *Melaleuca alternifolia* (tea tree) oil against dermatophytes and other filamentous fungi. *J Antimicrob Chemother* 2002; **50**:195–199.
18. Hammer KA, Carson CF, Riley TV. Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. *J Appl Microbiol* 2003;**95**:853–860.
19. Hammer KA, Dry L, Johnson M, Michalak EM, Carson CF,

Riley TV. Susceptibility of oral bacteria to *Melaleuca alternifolia* (tea tree) oil in vitro. *Oral Microbiol Immunol* 2003;**18**:389—392.

20. European standard EN 1276. *Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic, and industrial areas. Test method and requirements (phase 2, step 1).* European Committee for Standardization, Brussels. European standard EN, 1276; 1997.

21. European standard (pr)EN 12054. *Chemical disinfectants and antiseptics. Products for hygienic and surgical handrub and handwash. Bactericidal activity. Test method and requirements (phase 2, step 1).* European Committee for Standardization, Brussels. European standard EN 12054; 1995.

22. International Organization for Standardization. *ISO 4730 Oil of Melaleuca, terpinen-4-ol type (tea tree oil). Switzerland: International Organization for Standardization; 1996.*

23. Maillard J-Y, Messager S, Veillon R. Antimicrobial efficacy of biocides tested on skin using an 'ex-vivo' test. *J Hosp Infect* 1998;**40**:313—323.

24. Pittet D. Hand hygiene: improved standards and practice for hospital care. *Curr Opin Infect Dis* 2003;**16**:327—335.

25. Pittet D, Boyce JM. Hand hygiene and patient care: pursuing the Semmelweis legacy. *Lancet Infect Dis* 2001;**0**:9—20.

26. Hill RLR, Casewell MW. The in-vitro activity of povidone-iodine cream against *Staphylococcus aureus* and its bioavailability in nasal secretions. *J Hosp Infect* 2000;**45**:198—205.

27. Economou-Stamatelopoulou C, Papavassiliou J. Bactericidal activity of disinfectants against certain bacterial strains of *S. aureus. Bull Soc Pathol Exot Filiales* 1988;**81**:721—725.

28. Cardoso CL, Pereira HH, Zequim JC, Guilhermetti M. Effectiveness of hand-cleansing agents for removing *Acinetobacter baumannii* strain from contaminated hands. *Am J Infect Control* 1999;**27**:327—331.

29. Guilhermetti M, Hernandes SE, Fukushigue Y, Garcia LB, Cardoso CL. Effectiveness of hand-cleansing agents for removing methicillin-resistant *Staphylococcus aureus* from contaminated hands. *Infect Control Hosp Epidemiol* 2001;**22**:105—108.

30. Hobson DW, Woller W, Anderson L, Guthery E. Development and evaluation of a new alcohol-based surgical hand scrub formulation with persistent antimicrobial characteristics and brushless application. *Am J Infect Control* 1998;**26**:507—512.

31. Nishioka K, Seguchi T, Yasuno H, Yamamoto T, Tominaga K. The results of ingredient patch testing in contact dermatitis elicited by povidone-iodine preparations. *Contact Dermatitis* 2000;**42**:90—94.

32. Walsh SE, Maillard J-Y, Russell AD, Catrenich CE, Charbonneau DL, Bartolo RG. Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria. *J Appl Microbiol* 2003;**94**:240—247.

33. Hammer KA, Carson CF, Riley TV. Influence of organic matter, cations and surfactants on the antimicrobial activity of *Melaleuca alternifolia* (tea tree) oil in vitro. *J Appl Microbiol* 1999;**86**:446—452.

34. Cox SD, Mann CM, Markham JL. Interactions between components of the essential oil of *Melaleuca alternifolia. J Appl Microbiol* 2001;**91**:492—497.

35. Sakuragi T, Yanagisawa K, Dan K. Bactericidal activity of skin disinfectants on methicillin-resistant *Staphylococcus aureus. Anesth Analg* 1995;**81**:555—558.

36. Herrera D, Roldan S, Santacruz I, Santos S, Masdevall M, Sanz M. Differences in antimicrobial activity of four commercial 0.12% chlorhexidine mouthrinse formulations: an in vitro contact test and salivary bacterial counts study. *J Clin Periodontol* 2003;**30**:307—314.

37. Messager S, Goddard P, Dettmar PW, Maillard J-Y. Determination of the antibacterial efficacy of several antiseptics tested on skin by an 'ex-vivo' test. *J Med Microbiol* 2001;**50**:284—292.

38. Jeng DK, Severin JE. Povidone iodine gel alcohol: a 30-second, onetime application preoperative skin preparation. *Am J Infect Control* 1998;**26**:488—494.

39. Kampf G, Hofer M, Wendt C. Efficacy of hand disinfectants against vancomycin-resistant enterococci in vitro. *J Hosp Infect* 1999;**42**:143—150.

40. Marchetti MG, Kampf G, Finzi G, Salvatorelli G. Evaluation of the bactericidal effect of five products for surgical hand disinfection according to prEN 12054 and prEN 12791. *J Hosp Infect* 2003;**54**:63—67.

41. Winnefeld M, Richard MM, Drancourt M, Grobb JJ. Skin tolerance and effectiveness of two hand decontamination procedures in everyday hospital use. *Br J Dermatol* 2000;**143**:546—550.

42. Parienti JJ, Thibon P, Heller R, *et al*. Antisepsie Chirurgicale des mains Study Group. Hand-rubbing with an aqueous alcoholic solution vs traditional surgical hand-scrubbing and 30-day surgical site infection rates: a randomized equivalence study. *JAMA* 2002;**288**:722—727.

43. Boyce JM, Kelliher S, Vallande N. Skin irritation and dryness associated with two hand hygiene regimens: soap and water handwashing versus hand antisepsis with an alcoholic hand gel. *Infect Control Hosp Epidemiol* 2000;**21**:442—448.

44. Kampf G, Muscatiello M. Dermal tolerance of Sterillium, a propanol-based hand rub. *J Hosp Infect* 2003;**55**:295—298.

45. Reybrouck G. Evaluation of antimicrobial and antifungal efficacy. In: Russell AD, Hugo WB, Ayliffe GAJ, editors. *Principles and practice of disinfection, sterilization and preservation*. Oxford: Blackwell Scientific Publications; 1999. p. 124—144.

46. European standard EN 1499. *Chemical disinfectants and antiseptics. Hygienic handwash. Test method and requirements (phase 2/step 2).* European Committee for Standardization, Brussels. European standard EN, 1499; 1997.

47. European standard EN 1500. *Chemical disinfectants and antiseptics. Hygienic handrub. Test method and requirements (phase 2/step 2).* European Committee for Standardization, Brussels. European standard EN, 1500; 1997.