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 $>10^3$-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.
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 microorganisms.\(^1\)\(^8\)

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,\(^11\)\(^15\)\(^16\)\(^17\) 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 AnalR, 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 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.

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with the international standard for TTO (ISO 4730)\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsubscript{2}HPO\textsubscript{4} (BDH) and 0.24 g/L KH\textsubscript{2}PO\textsubscript{4} (BDH)]. The number of cfu/mL recovered was assessed by the drop counting method (DCM).\textsuperscript{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\textsuperscript{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\textsubscript{10} reductions $\leq$ 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\textsubscript{10} reduction factor of
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 °C ± 1 °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 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%
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 (log10 reduction \(\geq 5\)) 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 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

<table>
<thead>
<tr>
<th>Table III</th>
<th>List of products that achieved at least a 5 log10 reduction for various contact times and bacteria tested</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td><strong>Time</strong></td>
</tr>
<tr>
<td></td>
<td>1 min</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>100% AHR (P/C/D)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P/C/D)</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>100% HSW (C)</td>
</tr>
<tr>
<td></td>
<td>100% AHSW (P/C/D)</td>
</tr>
<tr>
<td></td>
<td>100% AHR (P/C/D)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P/C/D)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>5% TTO (P/C/D)</td>
</tr>
<tr>
<td></td>
<td>6% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>8% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>10% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>100% AHSW (C)</td>
</tr>
<tr>
<td></td>
<td>100% AHR (P/C)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P/C/D)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>4% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>6% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>8% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>10% TTO (P)</td>
</tr>
<tr>
<td></td>
<td>100% AHR (P/C)</td>
</tr>
<tr>
<td></td>
<td>7.5% PVI (P/C/D)</td>
</tr>
</tbody>
</table>

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.
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
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,
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* and *P. aeruginosa* (Figures 3 and 4). 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) (Figure 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* 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).

**AHR.**
The 100% AHR had good activity (log10 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>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>Contact time</th>
<th>Staphylococcus aureus log10 reductions</th>
<th>Acinetobacter baumannii log10 reductions</th>
<th>Escherichia coli log10 reductions</th>
<th>Pseudomonas aeruginosa log10 reductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% TTO</td>
<td>1 min</td>
<td>0.19 (0.36)*</td>
<td>0.13 (0.39)</td>
<td>4.38 (1.25)</td>
<td>0.22 (0.25)</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>0.92 (0.63)</td>
<td>0.85 (0.41)</td>
<td>5.26 (0.11)</td>
<td>0.93 (0.58)</td>
</tr>
<tr>
<td>2% TTO</td>
<td>1 min</td>
<td>0.40 (0.43)</td>
<td>-0.01 (0.06)</td>
<td>4.60 (0.93)</td>
<td>0.64 (0.21)</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.39 (0.64)</td>
<td>1.83 (0.39)</td>
<td>4.84 (0.93)</td>
<td>2.24 (0.60)</td>
</tr>
<tr>
<td>4% TTO</td>
<td>1 min</td>
<td>0.61 (0.53)</td>
<td>0.53 (0.41)</td>
<td>4.77 (1.05)</td>
<td>2.05 (0.48)</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.24 (0.74)</td>
<td>2.44 (0.52)</td>
<td>5.26 (1.20)</td>
<td>5.02 (0.91)</td>
</tr>
<tr>
<td>5% TTO</td>
<td>1 min</td>
<td>0.88 (0.58)</td>
<td>0.87 (0.43)</td>
<td>5.26 (1.20)</td>
<td>1.76 (0.62)</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.80 (0.45)</td>
<td>3.81 (0.34)</td>
<td>5.26 (1.20)</td>
<td>4.02 (0.51)</td>
</tr>
<tr>
<td>6% TTO</td>
<td>1 min</td>
<td>0.26 (0.41)</td>
<td>1.11 (1.08)</td>
<td>5.26 (1.20)</td>
<td>5.12 (0.34)</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>0.95 (0.71)</td>
<td>3.75 (1.43)</td>
<td>5.26 (1.20)</td>
<td>4.46 (0.57)</td>
</tr>
<tr>
<td>8% TTO</td>
<td>1 min</td>
<td>0.32 (0.10)</td>
<td>1.68 (0.94)</td>
<td>5.26 (1.20)</td>
<td>5.12 (0.34)</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.16 (1.03)</td>
<td>4.72 (0.89)</td>
<td>5.26 (1.20)</td>
<td>5.29 (0.19)</td>
</tr>
<tr>
<td>10% TTO</td>
<td>1 min</td>
<td>0.80 (0.38)</td>
<td>3.04 (1.12)</td>
<td>5.26 (1.20)</td>
<td>5.12 (0.34)</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>1.89 (0.60)</td>
<td>4.84 (0.68)</td>
<td>5.26 (1.20)</td>
<td>5.29 (0.19)</td>
</tr>
</tbody>
</table>

TTO, tea tree oil.

* Log10 reductions±SD.
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

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**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*.
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 $\geq 4.52$ in all bacteria within a 1-min contact time (Figure 6). The AHSW tested at 55% achieved a $\log_{10}$ reduction $\geq 2.52$ against *A. baumannii*, *E. coli* and *P. aeruginosa*, while 4.12% PVI also complied with the standard method by achieving a $\log_{10}$ reduction $\geq 3.5$ 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. However, compliance with hand-hygiene guidelines is low 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. 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. 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 $\geq 5$ 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\textsuperscript{26} who reported that 5% PVI induced a \( \log_{10} \) reduction of 5 in \textit{S. aureus} cell numbers after a 1-min contact time, and those obtained by Maillard et al.\textsuperscript{23} who showed a \( \log_{10} \) reduction >5 in \textit{E. coli} and \textit{P. aeruginosa} cell numbers after a 1-min contact time with 2% PVI. Economou-Stamatelopoulou and Papa-vasiliou\textsuperscript{27} also showed that the activity of 5% PVI was not significantly affected by the addition of interfering substances in suspension when assessed against \textit{S. aureus}. In-vivo studies have shown 10% PVI to be the most effective agent, with 70% ethyl alcohol, to remove \textit{A. baumannii}\textsuperscript{28} and \textit{S. aureus}\textsuperscript{29} from the contaminated hands of volunteers. PVI is generally recommended for use in hospital settings as a surgical-scrub agent (7.5%)\textsuperscript{30} and a hand-cleansing agent (10%).\textsuperscript{28,29} However, PVI can irritate the skin\textsuperscript{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,\textsuperscript{14} and this finding might be used to encourage healthcare staff’s compliance with handwashing. In addition, Hammer et al.\textsuperscript{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 \textit{P. aeruginosa} and \textit{A. baumannii}. Walsh et al.\textsuperscript{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 \( \mu g/mL \) thymol against \textit{E. coli} was reduced in dirty conditions, but the efficacy of thymol against \textit{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 \textit{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 \textit{P. aeruginosa} cells greater than 4.\textsuperscript{32} The activity of 100% AHSW and 100% AHR against \textit{E. coli} and \textit{P. aeruginosa} was reduced in dirty conditions, although this reduction was not significant. Hammer et al.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{35,36} Triclosan\textsuperscript{37} and PVI.\textsuperscript{38} Furthermore, the TTO-containing formulations assessed in this study also performed well according to the draft European standard method prEN 12054\textsuperscript{41} for the assessment of hygienic handrub and handwash solutions. The AHSW successfully passed the European recommendations after a 1-min contact time against \textit{E. coli}, \textit{A. baumannii} and \textit{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,\textsuperscript{39,40} and are more ‘skin-friendly’ and hence more tolerated by healthcare staff.\textsuperscript{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.\textsuperscript{3,25} In addition, they have been reported to cause less irritation and
skin dryness than commonly used soaps and antiseptics. 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, and thus active compounds should be assessed using practical tests. Suspension tests are indeed less severe than capacity tests and practical tests. 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 ≥ 5% also complied with EN 1276 by achieving log10 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. The hygienic hand washes are also being assessed in a handwashing study based on European standard EN 1499. Further studies could also include the testing of the TTO-containing AHR using European standard EN 1500 for the evaluation of hygienic hand rubs.

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