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Antimicrobial efficacy of silver ions in combination with tea tree oil against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*

W.L. Low ^a, C. Martin ^{b,c}, D.J. Hill ^d, M.A. Kenward ^{a,b,*}

^a *Research Centre for Applied Science, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1LY, UK*

^b *Department of Pharmacy, School of Applied Science, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1LY, UK*

^c *Research Institute in Healthcare Science, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1LY, UK*

^d *Department of Biology, Geography and the Environment, School of Applied Science, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1LY, UK*

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* Corresponding author. Present address: Department of Pharmacy, School of Applied Science, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1LY, UK. Tel.: +44 1902 322 347.

E-mail address: M.A.Kenward@wlv.ac.uk (M.A. Kenward).

ABSTRACT

Tea tree oil (TTO) and silver ions (Ag^+), either alone or in combination with other antimicrobial compounds, have been used in the treatment of topical infections. However, there appears to be little data on the efficacy of TTO combined with silver in the absence of any other agents. TTO and Ag^+ were added, alone and in combination, to suspension cultures of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. Treatment of these cultures with TTO and Ag^+ at sub-minimal lethal concentrations resulted in an enhanced loss of viability compared with treatment with individual agents. The order of sensitivity to the combined agents was *P. aeruginosa* > *S. aureus* > *C. albicans*. The fractional lethal concentration index (FLCI) showed that these combinations of TTO and Ag^+ exerted a synergistic effect against *P. aeruginosa* (FLCI = 0.263) and an indifferent effect against *S. aureus* and *C. albicans* (FLCI = 0.663 and 1.197, respectively). The results indicate that combining these antimicrobial agents may be useful in decreasing the concentration of antimicrobial agents required to achieve an effective reduction in opportunistic pathogenic microorganisms that typically infect wounds.

1. Introduction

The extensive use of conventional antibiotics and synthetic antimicrobial drugs has resulted in the widespread emergence of resistant microorganisms. This has led to renewed interest in the use of alternative compounds and/or pre-antibiotic agents, especially those with multiple target sites, in a bid to limit the risk of developing microbial resistance. Such antimicrobial agents include tea tree oil (TTO) and silver ions (Ag^+), both of which display a broad spectrum of activity.

The mode of action of Ag^+ involves inactivation of membrane-bound proteins, interference with the solute and electron transport systems, and inhibition of respiratory enzymes to promote the generation of reactive oxygen species [1–3]. Penetration of Ag^+ into cells may also impair cell replication processes. Binding of Ag^+ to DNA molecules causes stress and coiling of DNA into a condensed structure that lacks the capacity for replication [2], thus leading to inhibition of vital cell functions.

TTO has been shown to have broad-spectrum antibacterial, antifungal, antiviral and anti-inflammatory activities [4–6]. The major component of TTO, terpinen-4-ol, is primarily responsible for its antimicrobial properties [4,7]. TTO preferentially partitions into biological membranes, causing bilayer expansion that impairs membrane integrity, inhibits membrane-embedded enzymes and increases membrane fluidity with subsequent leakage of intracellular components [8,9].

In addition, both agents have beneficial effects in the wound healing process [1,4]. At wound sites, Ag^+ induce the synthesis of epithelial cell matrix metalloproteinases to inhibit the activity of collagenase and increase cell metabolism and uptake of key trace elements (zinc and copper). This also promotes RNA and DNA synthesis, cell proliferation, epithelialisation and tissue repair [1,10]. Similarly, TTO suppresses monocyte production of inflammatory mediators such as tumour necrosis factor- α , interleukin (IL)- 1β , IL-8, IL-10 and prostaglandin E_2 [5], thus reducing oxidative damage to cells.

The versatility of silver and TTO against a wide range of microorganisms might be used to improve the current treatment strategies for various chronic wound infections, e.g. using lower concentrations of the agents in combination to produce an enhanced, preferably synergistic, effect.

Whilst TTO and Ag^+ are active in their own right, both have been combined with other antimicrobial agents in the treatment of skin infections, e.g. TTO–chlorhexidine [111], silver sulfadiazine (SSD), SSD–cerium nitrate and SSD–chlorhexidine [1]. However Ag^+ and TTO do not appear to have been used together. Therefore, the aim of this study was to investigate the in vitro antimicrobial efficacy of Ag^+ alone [using silver nitrate (AgNO_3)] and in combination with TTO against representative skin pathogens, namely *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*.

2. Materials and methods

Tryptone soya broth (TSB), tryptone soya agar (TSA), malt extract broth (MEB) and malt extract agar (MEA) (Lab M, Lancaster, UK) were prepared according to manufacturer's recommendations. AgNO₃ (99.85% purity; Acros Organics, Geel, Belgium) and pure TTO (ISO 4730:2004 specification; Aromatique Essential & Perfume Oils, Birkenhead, UK) were used as received. Silver solutions were freshly prepared in sterile distilled water for each experiment. Contamination checks were performed both for Ag⁺ solution and TTO by plating 100 µL samples on TSA and MEA and revealed no microbial growth after 24 h of incubation at 37 °C.

2.1. Preparation of inocula

Freeze-dried stock cultures of *P. aeruginosa* (NCIB 8295), *S. aureus* (NCIB 6571) and *C. albicans* (NCYC 854) from the University of Wolverhampton collection were reconstituted in appropriate sterile broth. Cultures were resuscitated by incubation at 37 °C (*P. aeruginosa* and *S. aureus* for 24 h and *C. albicans* for 48 h) in aerobic orbital shakers operating at 50 cycles/min (Series 25; New Brunswick Scientific Co. Inc., Edison, NJ). Prior to experimental use, overnight broth cultures were diluted with sterile broth to give a starter inoculum culture of ca. 1×10^7 colony-forming units (CFU)/mL.

2.2. Determination of the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC)

Serial doubling dilutions of Ag^+ and TTO were prepared aseptically using TSB or MEB as appropriate. Each dilution was inoculated with 50 μL of starter culture to give a final concentration of between 1×10^5 and 1×10^6 CFU in 5 mL per tube. Following incubation for 18 h at 37 °C, the MIC was taken as the lowest concentration with no visible growth (turbidity). Samples from concentrations with no visible growth were plated on equivalent agar media and the MLC was determined as the lowest concentration showing no visible colony formation after incubation for 18 h at 37 °C. When control tubes showed turbidity due to the agent per se, all tubes in the test series were plated to determine the MLC. All experiments were performed in triplicate and the results shown represent the mean.

2.3. Chequerboard assay to determine the antimicrobial activity of TTO and Ag^+ against the studied microorganisms

The antimicrobial action of both agents in combination was determined using the chequerboard assay as described in Karpanen et al. [11] with the following concentration ranges: *C. albicans*, 0.05–0.25% v/v TTO and 1×10^{-4} – 1×10^{-3} % w/v Ag^+ ; *S. aureus*, 0.4–2.0% v/v TTO and 5×10^{-4} – 8×10^{-3} % w/v Ag^+ ; and *P. aeruginosa*, 1.0–16.0% v/v TTO and 5×10^{-4} – 2.5×10^{-3} % w/v Ag^+ .

The MLC from the chequerboard assay was used to calculate the fractional lethal concentration (FLC) and FLC index (FLCI) according to the below formulae:

$$\text{FLC} = \frac{\text{MLC of antimicrobial agent in combination}}{\text{MLC of antimicrobial agent alone}}$$

$$\text{FLCI} = \text{FLC of agent 1} + \text{FLC of agent 2}$$

On the basis of the FLCI, the interaction of the agents was identified as synergistic ($\text{FLCI} \leq 0.5$), indifferent ($\text{FLCI} > 0.5-4.0$) or antagonistic ($\text{FLCI} > 4.0$) [11].

2.4. Time-kill study

Test flasks received 90 mL of broth containing sufficient agent to achieve the MLC upon addition of inoculum culture (10 mL). Following inoculation, the final cell concentration ranged between 1×10^5 and 1×10^6 CFU/mL. Control flasks containing TSB or MEB were similarly prepared by substituting sterile distilled water for AgNO_3 and liquid paraffin for TTO. All flasks were prepared in triplicate and were incubated at 37 °C in a shaking water-bath oscillating at 50 cycles/min (Mickle Laboratory Engineering Co. Ltd., Guildford, UK). The initial (time zero) viable count of each culture was determined from colony counts obtained using the Miles and Misra technique following overnight incubation at 37 °C. Seven further samples were then taken, first at 15 min, then every 30 min up to 2.5 h, and finally at 24 h post inoculation.

3. Results

3.1. Minimum inhibitory concentration and minimum lethal concentration

The order of sensitivity to TTO (MIC; MLC) was *C. albicans* (0.25% v/v; 0.25% v/v) > *S. aureus* (1% v/v; 2% v/v) > *P. aeruginosa* (12.5% v/v; 16% v/v). Sensitivity to Ag⁺ was in the order *C. albicans* (6.4×10^{-4} % w/v; 6.4×10^{-4} % w/v) > *P. aeruginosa* (2.5×10^{-4} % w/v; 1.6×10^{-3} % w/v) > *S. aureus* (5.1×10^{-3} % w/v; 5.1×10^{-3} % w/v).

Selection of concentrations for both agents for the time–kill studies and determination of the FLCI were based on MLC data.

3.2. Fractional lethal concentration index

FLCI values (Table 1) showed the active combination concentration of agents used against *P. aeruginosa* to be clearly synergistic. Although the values for the combination used against *S. aureus* and *C. albicans* indicated indifferent interactions, both values were considerably lower than the antagonistic value of >4.0.

3.3. Time–kill analysis

The response of *C. albicans* to TTO (Fig. 1A) showed a small decline in viability (0.5 of a log cycle) over the first 2.5 h compared with the control. However, viability was below the detection limit after 24 h exposure. In contrast, exposure to the MLC of Ag⁺ produced a rapid, three log cycle decline in viability at 2.5 h post exposure. Again, after 24 h the viability had declined to below the detection limit. When *C. albicans* was exposed to a combination of sub-MLC levels of each agent, viability rapidly declined to the limit of detection within 1 h.

The MLC of TTO against *S. aureus* (Fig. 1B) showed a clear three log cycle decline in viability over the first 2.5 h of exposure, whilst the MLC of Ag⁺ showed a similar effect but with only a two log cycle reduction. In each case, after 24 h exposure viability was below the detectable limit. In contrast, the combination of these agents at sub-MLC levels resulted in a dramatic loss of viability, reaching the limit of detection within 30 min.

Compared with the control culture, viability of *P. aeruginosa* was reduced by three log cycles in the first 2.5 h of exposure to the MLC of TTO (Fig. 1C); viability was below the detectable limit after 24 h exposure. When exposed to the MLC of Ag⁺, viability of *P. aeruginosa* fell rapidly to the detection limit after 1.5 h exposure.

Combined sub-MLC levels of each agent resulted in viability dropping to below the detection limit within 15 min.

All three organisms therefore show a similar trend, where exposure to combined TTO and Ag⁺ at sub-MLCs results in a rapid and dramatic loss of viability within 1 h of exposure compared with the individual agents at the MLC. The order of sensitivity to the combined agents was *P. aeruginosa* > *S. aureus* > *C. albicans*, which mirrors the FLCI value derived from the checkerboard assay results (Table 1). In addition, viability of all three organisms was below the detectable limit after 24 h exposure irrespective of the concentration or combination of agents used.

4. Discussion

The activity of both agents against the studied microorganisms demonstrated their ability to control the proliferation of common opportunistic skin pathogens in vitro.

The MICs/MLCs of TTO obtained in this experiment (Table 2) are comparable with the range of results reviewed by Carson et al. [4].

The MLC values observed for TTO against *P. aeruginosa* are marginally greater than, but are still consistent with, the range reported by Carson et al. [4]. The values are consistent with the known low permeability of the outer membrane to TTO [12] and the multidrug efflux system-mediated tolerance of *P. aeruginosa* to TTO [8,13]. The pattern of the time–kill curves presented here for *P. aeruginosa* and *S. aureus* are similar to those reported by May et al. [7] for clinical isolates treated with TTO.

Both *P. aeruginosa* and *S. aureus* are capable of forming clusters of cells in suspension and as biofilms [6,14]. The less rapid decline in CFU/mL both for *P. aeruginosa* and *S. aureus* when treated with TTO or Ag⁺ might indicate the possibility of the disaggregation of cell clusters by the antimicrobial agent(s), masking the true rate of decline in viable cell numbers.

The observed variation in activity of the agents against each culture may be due to structural differences between the microorganisms [2,15]. The different interactions, permeability, and diffusion of hydrophobic (TTO) and hydrophilic (Ag⁺ and some components of TTO) agents across the cell membrane may influence the extent of the antimicrobial effect. Hydrophobic components can usually diffuse across lipid

bilayers easily, whereas hydrophilic agents are often transported passively via channels present in the membranes [9]. In the case of *P. aeruginosa* (Gram-negative), the presence of lipopolysaccharides in the outer membrane protects against penetration by hydrophobic antimicrobial agents (TTO); thus, higher amounts of agent are required compared with *S. aureus* (Gram-positive) and *C. albicans* that lack an outer membrane. This observation is supported by a recent report on the resistance of *P. aeruginosa* to various antimicrobial agents, including TTO [8]. The greater sensitivity of *P. aeruginosa* to Ag^+ compared with *S. aureus* and *C. albicans* is consistent with the ions entering the periplasmic space via outer membrane porins rather than having to diffuse through the cell wall matrix.

TTO and Ag^+ express their antimicrobial properties when the agents react with the cell membrane and/or diffuse across to intracellular targets [2,8]. Thus, permeability of the membrane to Ag^+ will influence the ability of this agent to reach its intracellular targets. Cell membranes offer a hydrophobic environment that attracts lipophilic compounds, such as the monoterpenes in TTO, which weaken the membrane. This may increase the ability of Ag^+ to be transported into the cell to deactivate vital enzymes as well as denaturing DNA and RNA. In such conditions, the microbial cell may face a double killing effect that could give rise to the observed enhanced lethal effect when both agents are employed at sub-MLC levels.

Treatment of the three microorganisms with combined agents showed an increase in microbicidal activity as measured by MLC studies. In the case of *P. aeruginosa*, this increase was shown to be significantly synergistic ($\text{FLCI} \leq 0.5$), although the FLCIs for the combinations against *C. albicans* and *S. aureus* were within the 'indifferent'

range. The FLCI calculations are based on endpoint MLC data collected after 24 h exposure to the agents. However, when time–kill results are considered, all three organisms show an enhanced response to the combinations at an early stage of exposure, with a reduction in CFU/mL to the lowest detectable limit between 15 min and 60 min of treatment. This study as a whole suggests that a combination of TTO with Ag⁺ may prove effective against common Gram-positive, Gram-negative and fungal microorganisms associated with wound infections.

5. Conclusion

The versatility of these agent(s) against a range of microorganisms owing to their multiple target sites might be used to improve current treatment strategies for chronic wound infections. Despite the effectiveness of combined treatment, the toxicity of the individual agents towards human host cells should also be carefully considered. These results provide a basis for combined preparations to be explored as a means of enhancing the efficacy of these agents whilst minimising rapid development of resistance to single antimicrobials. The aims of our future work are to develop controlled, targeted-release formulations of TTO and/or Ag⁺ to facilitate the delivery of lower amounts of agent, at concentrations less toxic to the patient whilst still maintaining efficient treatment of infected wounds.

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Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Time–kill curves of (A) *Candida albicans* (NCYC 854), (B) *Staphylococcus aureus* (NCIB 6571) and (C) *Pseudomonas aeruginosa* (NCIB 8295) exposed to tea tree oil (TTO), silver ions (Ag^+) or TTO + Ag^+ at the indicated concentrations or to neither agent (control). Data represent the mean of three replicates \pm standard deviation. The lowest detectable limit was 1.69 log cycles or 50 colony-forming units (CFU)/mL.

Table 1

Antimicrobial activities of combined tea tree oil (TTO) and silver ions (Ag⁺) against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*

Microorganism	MLC of TTO (% v/v) in combination/alone	FLC of TTO	MLC of Ag ⁺ (% w/v) in combination/alone	FLC of Ag ⁺	FLCI	Inference
<i>C. albicans</i>	0.1/0.25	0.4	$5.1 \times 10^{-4}/6.4 \times 10^{-4}$	0.7969	1.1969	Indifferent
<i>S. aureus</i>	1.2/2	0.6	$3.2 \times 10^{-4}/5.1 \times 10^{-3}$	0.0627	0.6627	Indifferent
<i>P. aeruginosa</i>	1/16	0.0625	$3.2 \times 10^{-4}/1.6 \times 10^{-3}$	0.2	0.2625	Synergistic

MLC, minimum lethal concentration; FLC, fractional lethal concentration; FLCI, fractional lethal concentration index.

Table 2

Summary of correlation between minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of tea tree oil (TTO) in this experiment compared with the reported literature

Microorganism	% TTO (v/v) in this experiment		% TTO (v/v) from Carson et al. [4]	
	MIC	MLC	MIC	MLC
<i>Candida albicans</i>	0.25–1	0.25	0.03–0.5	0.12–2
<i>Staphylococcus aureus</i>	1–2	2	0.5–1.25	1–2
<i>Pseudomonas aeruginosa</i>	12.5–16	16	1–8	2 to >8

