Antimicrobial Agents and Chemotherapy

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Antimicrob. Agents Chemother. **2012, 56(2):909. DOI: 10.1128/AAC.05741-11.**
Published Ahead of Print 14 November 2011.

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Effects of *Melaleuca alternifolia* (Tea Tree) Essential Oil and the Major Monoterpene Component Terpinen-4-ol on the Development of Single- and Multistep Antibiotic Resistance and Antimicrobial Susceptibility

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This study examined the effect of subinhibitory *Melaleuca alternifolia* (tea tree) essential oil on the development of antibiotic resistance in *Staphylococcus aureus* and *Escherichia coli*. Frequencies of single-step antibiotic-resistant mutants were determined by inoculating bacteria cultured with or without subinhibitory tea tree oil onto agar containing 2 to 8 times the MIC of each antibiotic and with or without tea tree oil. Whereas most differences in resistance frequencies were relatively minor, the combination of kanamycin and tea tree oil yielded approximately 10-fold fewer resistant *E. coli* mutants than kanamycin alone. The development of multistep antibiotic resistance in the presence of tea tree oil or terpinen-4-ol was examined by culturing *S. aureus* and *E. coli* isolates daily with antibiotic alone, antibiotic with tea tree oil, and antibiotic with terpinen-4-ol for 6 days. Median MICs for each antibiotic alone increased 4- to 16-fold by day 6. Subinhibitory tea tree oil or terpinen-4-ol did not greatly alter results, with day 6 median MICs being either the same as or one concentration different from those for antibiotic alone. For tea tree oil and terpinen-4-ol alone, day 6 median MICs had increased 4-fold for *S. aureus* ($n = 18$) and 2-fold for *E. coli* ($n = 18$) from baseline values. Lastly, few significant changes in antimicrobial susceptibility were seen for *S. aureus* and *S. epidermidis* isolates that had been serially subcultured 14 to 22 times with subinhibitory terpinen-4-ol. Overall, these data indicate that tea tree oil and terpinen-4-ol have little impact on the development of antimicrobial resistance and susceptibility.

Plants have long been recognized as a valuable source of medicinal agents. In particular, secondary plant metabolites such as essential oils have been used throughout history for therapeutic purposes. The essential oil that is steam distilled from the Australian native plant *Melaleuca alternifolia* (Myrtaceae), also known as melaleuca oil or tea tree oil (TTO), is used topically for its antimicrobial and anti-inflammatory effects (5). The oil contains predominantly monoterpenes and related alcohols, and its composition is regulated by the international standard ISO 4730:2004 (20). MICs of tea tree oil are typically between 0.125 and 2% (vol/vol) (5, 9), and bactericidal activity is largely attributable to nonspecific membrane effects (6, 9). Clinical studies with tea tree oil products have shown efficacy for a range of superficial infections, including acne, cold sores, tinea, and oral candidiasis, as well as for the decolonization of methicillin-resistant *Staphylococcus aureus* carriage (5). Irritant reactions and contact allergy have been reported infrequently and can be minimized by avoiding the use of neat oil and storing oil correctly (5).

Two recent studies suggested that several bacteria that had been exposed to tea tree oil subsequently were less susceptible to antibiotics *in vitro* (23, 24). Although decreases in antibiotic susceptibility were transient, this nonetheless raises concerns that tea tree oil hinders the effectiveness of conventional antibiotics by either reducing susceptibility or influencing the development of resistance. This is particularly important if tea tree oil is to become more widely used in hospital environments or in long-term care facilities, such as for the decolonization of MRSA carriers (3, 11, 30). The purpose of this study therefore was to examine whether tea tree oil or its major component, terpinen-4-ol (T4ol), influences the development of *de novo* antibiotic resistance in medically important bacteria.

MATERIALS AND METHODS

Bacteria and antimicrobials. Reference and clinical isolates of *Staphylococcus aureus* ($n = 18$), *Escherichia coli* ($n = 21$), and *Staphylococcus epidermidis* ($n = 1$), including antibiotic-resistant strains, were obtained from the Division of Microbiology and Infectious Diseases at PathWest Laboratory Medicine WA. References strains were *S. aureus* NCTC 6571, NCTC 29213, and ATCC 25923, *E. coli* NCTC 10418, ATCC 25922, ATCC 43889, ATCC 43894, and ATCC 11775, and *S. epidermidis* ATCC 12228. Ciprofloxacin, vancomycin, mupirocin, kanamycin, ampicillin, and rifampin were purchased from Sigma-Aldrich (St. Louis, MO). Benzalkonium chloride (>95% pure) and triclosan (Irgasan; ≥97%) were purchased from Fluka (Buchs, Switzerland). Terpinen-4-ol (97.0%) was obtained from Acros Organics (Geel, Belgium). Tea tree oil (batch A352) was provided by P. Guinane Pty. Ltd., Cudgen, New South Wales, Australia. The composition was determined by gas chromatography-mass spectrometry, which was performed by Diagnostic and Analytical Services Environmental Laboratory, Wollongbar, New South Wales, Australia, and complied with ISO 4730 (20). The major components of the oil were terpinen-4-ol (37.0%), γ-terpinene (18.6%), α-terpinene (10.0%), and

Received 16 September 2011 Returned for modification 10 October 2011
Accepted 7 November 2011
Published ahead of print 14 November 2011
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doi:10.1128/AAC.05741-11

1,8-cineole (3.6%). Solutions of tea tree oil and terpinen-4-ol (measured in %, vol/vol) were prepared daily and used within 2 h.

Single-step resistance studies. MICs for each antibiotic, tea tree oil, and terpinen-4-ol were determined by agar dilution using the Clinical and Laboratory Standards Institute method (8), with the inclusion of 0.5% Tween 20 in the agar as a solubilizer for the latter two antimicrobials. Inocula were prepared by culturing *E. coli* and *S. aureus* isolates overnight in trypticase soy broth (TSB) and then diluting them 1:10 into fresh TSB with 0.001% Tween 80, both without (treatments A and B) and with (treatments C and D) 0.03125% tea tree oil. This tea tree oil concentration was determined in preliminary growth curve experiments to be the highest concentration allowing approximately normal growth (data not shown). Cultures were incubated at 37°C with shaking until mid-late logarithmic phase. Cells then were collected, washed, and resuspended in 1/10 of the original volume in 0.85% saline. The cell suspension then was diluted in a series of 10-fold dilutions in 0.85% saline, and viable counts were performed on each cell suspension on Mueller-Hinton agar (MHA) both without (treatments A and C) and with tea tree oil (treatments B and D).

Agar plates were prepared containing each antibiotic in 20 ml MHA with a final concentration of 0.5% (vol/vol) Tween 20. A second set was prepared in parallel containing antibiotic with tea tree oil. For *S. aureus*, final antibiotic concentrations were 2× MIC for ciprofloxacin and vancomycin and 8× MIC for mupirocin and rifampin. Where relevant, 0.25% (1/2× MIC) tea tree oil was included in the agar. For *E. coli*, final antibiotic concentrations were 2× MIC for kanamycin and ampicillin, 1× MIC for ciprofloxacin, and 8× MIC for rifampin. Agar contained 0.125% (1/4× MIC) tea tree oil. These tea tree oil concentrations were determined in preliminary experiments (data not shown). Plates containing antibiotic alone were stored for a maximum of 7 days at 4°C before use, whereas plates containing tea tree oil were prepared on the day of the experiment. Agar plates were inoculated by spreading 100-μl volumes from the appropriate dilution of cell suspension onto each agar plate. Plates then were incubated at 30 to 35°C for 24 to 72 h, and colonies (single-step mutants) were counted. Frequencies of resistance were calculated by dividing the number of mutants (in CFU/ml) by the number of CFU in the inoculum. The assay was repeated at least three times on separate occasions for each isolate and each antibiotic. Geometric means of resistance frequencies then were determined for each isolate.

Multistep resistance studies. Multistep resistance was selected for by using the CLSI broth microdilution method (8) with minor modifications. Briefly, a series of doubling dilutions of each antibiotic was prepared in Mueller-Hinton broth in triplicate in a 96-well microtiter tray. The first dilution series contained antibiotic alone, the second contained the antibiotic with a final concentration of 0.062% tea tree oil, and the third contained the antibiotic with a final concentration of 0.031% terpinen-4-ol. All wells contained a final concentration of 0.001% Tween 80 to enhance the solubility of tea tree oil/terpinen-4-ol. A minimum of 10 isolates of each species was examined per antibiotic. Additional microtiter trays containing doubling dilutions of tea tree oil or terpinen-4-ol alone also were prepared to determine whether susceptibility to either substance changed over the course of the assay. Each triplicate dilution series was inoculated with exponential-phase cultures adjusted to result in final inoculum concentrations of $\sim 5 \times 10^5$ CFU/ml. All trays were incubated for 24 h at 37°C with shaking at 120 rpm and examined visually. The MIC was recorded as the lowest concentration resulting in a significant decrease in growth. To perform the serial subculture, an aliquot of culture from the concentration immediately below the MIC (i.e., 1/2× MIC) was removed, diluted 1:5, and used to inoculate a fresh tray containing the identical combination of antibiotic with or without TTO or terpinen-4-ol prepared as described above. This procedure was repeated for a total of 6 days. The medians and geometric means of MICs obtained for each combination then were determined. If the median fell between dilution values, the higher of the two values was selected.

Effect of terpinen-4-ol serial passage on antimicrobial susceptibility. These experiments were conducted to (i) further attempt to induce terpinen-4-ol resistance by broth macrodilution and (ii) determine antibiotic susceptibility after serial subculture. Overnight cultures of the three test organisms, *S. aureus* NCTC 6571, *S. aureus* ATCC 25923, and *S. epidermidis* ATCC 12228, in TSB were diluted 1:10 into TSB containing 0.05% terpinen-4-ol with 0.001% Tween 80. Cultures were incubated at 37°C on a Stuart SF1 flask shaker (Bibby Scientific, Staffordshire, United Kingdom) with wrist-action shaking equivalent to 500 rpm for 24 h. Cultures then were diluted 1:10 into fresh TSB containing 0.1 and 0.2% terpinen-4-ol and were incubated as described above for 1 to 4 days. Bacteria from the highest concentration that was visibly turbid then were diluted into two fresh terpinen-4-ol solutions at the same concentration and a slightly higher concentration, using 0.1% increments. This process was repeated until organisms failed to grow. *S. aureus* ATCC 25923 also was cultured as described above with tea tree oil, and a control culture of TSB with 0.001% Tween 80 but without tea tree oil or terpinen-4-ol was maintained identically throughout in parallel (passaged control). The susceptibility of serially passaged isolates was determined by removing an aliquot from a serial-passage culture that had been incubated for no more than 24 h (control, tea tree oil, and/or terpinen-4-ol), collecting cells by centrifugation, washing them twice, and then resuspending them in 0.85% saline. The cell concentration was adjusted to approximately 10^8 CFU/ml, and susceptibility was determined by the broth microdilution method (8). Inocula for the nonpassaged control were prepared by culturing bacteria from a stock stored at −80°C onto blood agar, incubating overnight, then inoculating into TSB and culturing organisms until mid-exponential phase. MICs of all antimicrobial agents were determined according to CLSI criteria (8).

Statistical analyses. Frequencies of resistance data were first transformed to their corresponding $\log_{10}$ values. However, for ease of representation, frequencies are shown as the geometric means. Transformed resistance frequencies then were analyzed by a repeated-measure one-way analysis of variance (ANOVA) with the Bonferroni *post hoc* test ($P < 0.05$). MICs from the multistep experiments were log transformed (base 2) to approximate normal distributions. Log_2 values then were analyzed by repeated-measure one-way ANOVA with the Bonferroni *post hoc* test ($P < 0.05$). All statistical analyses were performed using GraphPad Prism (version 3.03) software, and differences were considered significant when $P \leq 0.05$.

RESULTS

Baseline MICs for *S. aureus* were the following: ciprofloxacin, 0.06 to >8 μg/ml; vancomycin, 0.5 to 2 μg/ml; mupirocin, 0.06 to 0.12 μg/ml; rifampin, 0.004 to 0.008 μg/ml; tea tree oil, 0.5%; and terpinen-4-ol, 0.25%. For *E. coli*, baseline MICs were ciprofloxacin, 0.008 to >32 μg/ml; kanamycin, 2 to >32 μg/ml; ampicillin, 1 to >32 μg/ml; rifampin, 4 to >64 μg/ml; tea tree oil, 0.25 to 0.5%; and terpinen-4-ol, 0.12 to 0.25%. Resistance frequencies for vancomycin and ciprofloxacin did not differ significantly in the presence and absence of tea tree oil for *S. aureus* (Table 1). For rifampin, significant differences were found between treatments B and C and for mupirocin between treatments A and B, B and C, and C and D. However, differences were minor, i.e., less than 1 log in magnitude. For *E. coli*, frequencies of resistance to rifampin did not differ significantly in the presence of tea tree oil. Kanamycin resistance frequencies differed significantly for all treatments with the exception of treatments A and C. Approximately 1 log fewer kanamycin-resistant mutants were detected when tea tree oil was present in the agar than when it was absent.

For multistep assays, MICs for *S. aureus* increased by more than double (4-fold) from the baseline for ciprofloxacin, mupirocin, and vancomycin alone after 2 to 4 days and on day 6 for TTO and terpinen-4-ol (alone) (Table 2). On day 6, median MICs for

TABLE 1 Frequencies of single-step antibiotic-resistant mutants occurring in the presence and absence of tea tree oil[a]

Organism and no. of isolates	Antibiotic	Fold increase in MIC	Frequency of mutants in: Control culture with: Antibiotic alone (treatment A)	Control culture with: Antibiotic + TTO (treatment B)	TTO culture with: Antibiotic alone (treatment C)	TTO culture with: Antibiotic + TTO (treatment D)	*P* value[b]
S. aureus							
10	RIF	8	8.9×10^{-8}	5.9×10^{-8}	1.1×10^{-7}	7.2×10^{-8}	0.0065[c]
10	MUP	8	7.3×10^{-8}	2.3×10^{-8}	9.9×10^{-8}	3.5×10^{-8}	0.0002[d]
7	VAN	2	2.4×10^{-7}	1.2×10^{-7}	3.4×10^{-7}	5.9×10^{-7}	0.1268
4	CIP	2	8.1×10^{-8}	5.9×10^{-8}	8.6×10^{-8}	3.7×10^{-8}	0.6725
E. coli							
10	RIF	8	3.7×10^{-8}	2.9×10^{-8}	4.3×10^{-8}	3.8×10^{-8}	0.2083
9	KAN	2	3.3×10^{-6}	2.0×10^{-7}	2.6×10^{-6}	3.1×10^{-7}	<0.0001[e]

[a] Values are the geometric means from 4 to 10 isolates. MUP, mupirocin; RIF, rifampin; VAN, vancomycin; CIP, ciprofloxacin; KAN, kanamycin; TTO, tea tree oil.
[b] *P* values were obtained by repeated-measure one-way ANOVA.
[c] Significant differences exist between treatments B and C ($P < 0.01$) (Bonferroni post test).
[d] Significant differences exist between treatments A and B ($P < 0.01$), B and C ($P < 0.001$), and C and D ($P < 0.05$).
[e] Significant differences exist between treatments A and B ($P < 0.001$), A and D ($P < 0.01$), B and C ($P < 0.001$), and C and D ($P < 0.01$).

antibiotic alone increased 4-fold for ciprofloxacin and vancomycin and 8-fold for mupirocin compared to MICs at day 1. The presence of TTO or terpinen-4-ol with antibiotic did not appear to greatly influence MICs, with median MICs being either identical or differing by one dilution only from antibiotic alone on all days. The only exception was mupirocin on day 5, where the median MIC in the presence of terpinen-4-ol was 4-fold that of mupirocin alone. However, at day 6 this difference was only 2-fold. The sta-

TABLE 2 *S. aureus* MICs of antibiotics (μg/ml) alone, antibiotics with or without tea tree oil (0.062%) or terpinen-4-ol (0.031%), and tea tree oil or terpinen-4-ol without antibiotic, determined by serial subculture[a]

Agent (no. of isolates) and parameter	Treatment	MIC on day: 1	2	3	4	5	6
CIP (10)							
Median	Alone	0.5	1	1	**2**	**2**	**2**
	With TTO	0.25	**1**	**1**	**2**	**2**	**2**
	With T4ol	0.25	0.5	**1**	**2**	**2**	**2**
GM	Alone	0.8	0.8	1.1	1.5	1.6	2.3
	With TTO	0.25	1.0	1.1	1.6	2.5	3.5
	With T4ol	0.2	0.6	1.0	1.5	1.7	2.3
MUP (11)							
Median	Alone	0.12	0.25	**0.5**	**1**	**1**	**1**
	With TTO	0.12	0.12	**0.5**	**0.5**	**0.5**	**1**
	With T4ol	0.12	0.12	**0.5**	**1**	**4**	**2**
GM	Alone	0.1	0.3	0.5	0.9	1.2	1.5
	With TTO	0.1	0.2	0.5	0.6	0.5	1.4
	With T4ol	0.1	0.2	0.5	0.9	1.9	3.1
VAN (12)							
Median	Alone	1	2	**4**	**8**	**4**	**4**
	With TTO	1	**4**	**4**	**8**	**4**	**4**
	With T4ol	1	**4**	**4**	**8**	**4**	**4**
GM	Alone	1.0	2.7	4.5	7.6	4.0	3.8
	With TTO	1.1	5.0	5.0	6.3	3.8	4.3
	With T4ol	0.9	4.0	4.0	9.5	3.6	5.0
Tea tree oil (18)							
Median	Alone	0.5	0.5	1	1	1	**2**
GM	Alone	0.5	0.7	1.0	0.9	1.0	1.7
Terpinen-4-ol (18)							
Median	Alone	0.25	0.25	0.25	0.5	0.5	**1**
GM	Alone	0.2	0.3	0.3	0.6	0.6	0.8

[a] GM, geometric means. Boldface type indicates that the MIC is more than double the baseline (day 1) value. Single underlining indicates that values differed significantly from antibiotic alone on that day. Double underlining indicates significant differences between tea tree oil and terpinen-4-ol treatments on that day.

TABLE 3 Summary of *E. coli* MICs of antibiotics (μg/ml) alone, antibiotics with tea tree oil (0.062%) or terpinen-4-ol (0.031%), and tea tree oil or terpinen-4-ol without antibiotics, determined by serial subculture[a]

Agent (no. of isolates) and parameter	Treatment	MIC on day:					
		1	2	3	4	5	6
CIP (12)							
Median	Alone	0.008	0.016	0.016	**0.03**	**0.03**	**0.12**
	With TTO	0.008	0.016	**0.03**	**0.06**	**0.06**	**0.12**
	With T4ol	0.008	0.016	**0.06**	**0.06**	**0.12**	**0.12**
GM	Alone	0.010	0.025	0.034	0.070	0.070	0.140
	With TTO	0.007	0.014	0.029	0.045	0.064	0.122
	With T4ol	0.009	0.018	0.048	0.078	0.100	0.147
KAN (11)							
Median	Alone	8	**32**	16	**64**	**64**	**128**
	With TTO	4	**16**	**32**	**32**	**32**	**64**
	+With T4ol	4	**16**	**32**	**32**	**32**	**64**
GM	Alone	6.7	45.3	20.2	60.4	50.8	107.6
	With TTO	4.8	12.7	33.9	38.1	42.7	47.9
	With T4ol	2.5	15.1	28.5	33.9	32.0	53.8
AMP (10)							
Median	Alone	2	4	**8**	**8**	**16**	**16**
	With TTO	2	4	**8**	**8**	**16**	**16**
	With T4ol	2	4	**8**	**8**	**16**	**32**
GM	Alone	2.1	4.9	8.0	10.6	11.3	16.0
	With TTO	2.1	4.6	8.6	9.8	12.1	12.1
	With T4ol	2.1	4.9	11.3	11.3	17.1	24.3
Tea tree oil (18)							
Median	Alone	0.5	1	1	1	1	1
GM	Alone	0.65	0.73	0.96	1.00	1.12	0.96
Terpinen-4-ol (18)							
Median	Alone	0.12	0.25	0.25	0.25	0.25	0.25
GM	Alone	0.13	0.17	0.22	0.29	0.25	0.26

[a] GM, geometric means. Boldface type indicates that the MIC is more than double the baseline (day 1) value. Single underlining indicates that values differed significantly from antibiotic alone on that day. Double underlining indicates significant differences between tea tree oil and terpinen-4ol treatments on that day.

tistical analysis of MICs obtained on each day under the three different conditions (antibiotic alone, with tea tree oil, or with terpinen-4-ol) demonstrated significant differences for ciprofloxacin on days 1 ($P < 0.0001$) and 2 ($P = 0.02$), for vancomycin on days 2 ($P < 0.0001$), 4 ($P = 0.0017$), and 6 ($P < 0.0001$), and for mupirocin on day 2 ($P = 0.0082$).

For *E. coli*, increases in the MIC of more than two doubling dilutions occurred for all three antibiotics alone on days 2 to 3 (Table 3). Increases in median MICs from days 1 to 6 for antibiotic alone were 16-fold for ciprofloxacin and kanamycin and 8-fold for ampicillin. Similarly to *S. aureus*, the presence of TTO or terpinen-4-ol with antibiotic did not appear to greatly influence MICs, with median MICs obtained under the three conditions being either the same or differing by one dilution only on each day. The exception was ciprofloxacin with terpinen-4-ol, where the median MIC was 4-fold higher than that of ciprofloxacin alone on days 3 and 5. The analysis of MICs showed significant differences between the three conditions for ciprofloxacin on day 1 ($P < 0.0001$), for kanamycin on days 1 ($P < 0.0001$), 2 ($P < 0.0001$), and 6 ($P = 0.0288$), and for ampicillin on day 6 ($P = 0.0383$). For tea tree oil and terpinen-4-ol alone, the median MIC increased 2-fold during the 6 days.

Lastly, using a macrodilution method, *S. aureus* strains did not grow consistently in concentrations greater than 0.1% terpinen-4-ol after 18 to 20 passages, demonstrating that resistance to terpinen-4ol could not be induced *in vitro* (Table 4). Similarly, *S. epidermidis* ATCC 12228 would not grow at concentrations above 0.2% terpinen-4-ol, and *S. aureus* ATCC 25923 would not grow above 0.1% tea tree oil. Serial passage with terpinen-4-ol resulted in few changes in antimicrobial susceptibility (Table 1). Changes in MICs of two or more dilutions were evident for ciprofloxacin, gentamicin, tetracycline, and benzalkonium chloride only. However, with the exception of benzalkonium chloride and *S. aureus* ATCC 25923, differences were not observed consistently for every passage number. The susceptibility of multiply passaged *S. aureus* NCTC 6571 to tetracycline reverted to 0.25 μg/ml after the organism was stored at −80°C and then recultured. MICs for *S. aureus* ATCC 25923 passaged in 0.1% TTO did not differ by more than 1 dilution from that of the control. Passaging in TSB alone did not produce significant changes in MICs, as susceptibility data for the passaged and nonpassaged controls did not vary by more than 1 dilution for all three strains (data not shown).

DISCUSSION

There are many examples in the literature of the presence of a second antimicrobial agent or nonantibiotic drug preventing or

TABLE 4 MICs of antibiotics (μg/ml), biocides (μg/ml), and tea tree oil and terpinen-4-ol (%, vol/vol) for three *Staphylococcus* strains serially subcultured with terpinen-4-ol or tea tree oil[a]

	S. aureus NCTC 6571			*S. aureus* ATCC 25923				*S. epidermidis* ATCC 12228		
		MIC			MIC				MIC	
Agent	Passage no.	Control	With 0.1% T4ol	Passage no.	Control	With 0.1% T4ol	With 0.1% TTO	Passage no.	Control	With 0.2% T4ol
AMX	19	0.12	0.06	18	0.12	0.12	0.12	14	1	0.5
	22	0.25	0.25	20	0.5	0.25		17	1	0.5
CIP	17	0.25	**0.06**	16	0.12	0.12	0.25	14	0.25	0.25
	19	0.12	0.06	18	0.25	**0.06**	0.12	17	0.5	0.25
	22	0.12	0.06	20	0.25	**0.06**				
GEN	17	1	2	16	0.5	0.25	0.5	14	0.12	**0.5**
	19	1	1	18	0.25	0.12	0.12	17	0.25	0.25
	22	1	2	20	0.25	0.25				
TET	17	0.12	0.06	16	0.12	0.12	0.25	14	0.5	0.5
	19	0.25	**<0.03**	18	0.25	0.12	0.25	17	1	0.5
	22	0.12	0.06	20	0.25	0.12				
VAN	17	1	1	16	2	2	2	14	4	4
	19	1	0.5	18	1	1	2	17	4	4
	22	0.5	0.25	20	1	1				
Benzalkonium	17	0.5	1	16	2	**0.5**	2	14	2	1
Chloride	19	1	1	18	2	**0.5**	1	17	2	1
	22	1	1	20	2	**0.5**				
Triclosan	19	0.06	0.03	18	0.06	0.03	0.03	17	0.03	0.06
	22	0.12	0.12	20	0.25	0.12				
Tea tree oil	17	0.5	0.25	16	0.12	0.25	0.25	14	0.5	0.5
	19	1	0.5	18	0.5	0.5	0.5	17	0.5	0.5
	22	0.25	0.25	20	0.5	0.5				
Terpinen-4-ol	17	0.25	0.5	16	0.25	0.5	0.5	14	0.5	0.5
	19	0.5	0.5	18	0.5	0.5	0.25	17	0.5	0.5
	22	0.12	0.25	20	0.12	0.25				

[a] Boldface type indicates a difference in MIC of 4-fold or more for passaged and nonpassaged strains.

delaying the development of antibiotic resistance (22, 27). One of the best known is the treatment of tuberculosis with combinations of rifampin, isoniazid, pyrazinamide, and ethambutol (19, 29). At the other end of the spectrum, there are concerns that the overuse of antimicrobial agents such as biocides leads to increases in antibiotic resistance (15). These concerns relate to the use of disinfectants and antiseptics in the domestic environment and the theory that the increased and chronic exposure of bacteria to sublethal concentrations of biocide leads to tolerance, which may also confer tolerance to antibiotics. Since several biocides have multiple, nonspecific mechanisms of action, similarly to tea tree oil, this same concern could apply to the oil. Although decreased antibiotic susceptibility following biocide exposure has been demonstrated *in vitro* (4, 16), there is still debate as to what impact, if any, this has in clinical practice (17). The current study has demonstrated that tea tree oil has little impact on the development of antibiotic resistance, and that exposure to the major component terpinen-4-ol does not significantly alter antimicrobial susceptibility.

Frequencies of single-step antibiotic resistance were largely unaffected by either culturing with tea tree oil or combining antibiotic with tea tree oil. The exception was kanamycin, whereby *E. coli* resistance frequencies were consistently approximately 1 $\log_{10}$ lower when cultured on kanamycin agar with tea tree oil for both control cultures and tea tree oil cultures. Culturing with tea tree oil prior to determining resistance frequencies had no significant impact. Two possible explanations for the differences in resistance frequencies are that the tea tree oil is preventing mutations (and decreasing the overall mutation rate) or decreasing the survival of a small proportion of resistant mutants (no change in mutation rate). There is little evidence to support the first possibility, since (i) if this was the case we would expect more differences in mutation rates in the current study, and (ii) previous studies have shown that tea tree oil neither increases (12, 14) nor decreases (12) mutations using the bacterial reverse mutation assay. This therefore suggests that the decreased number of mutants is specific to kanamycin and its mechanism(s) of action and resistance. Aminoglycosides exert antibacterial action primarily by interfering with protein synthesis by binding to rRNA in the small subunit of the bacterial ribosome. Mechanisms of kanamycin resistance include

the reduction of intracellular antibiotic concentration (typically via efflux), the alteration of the target site (normally by spontaneous mutation), and enzymatic inactivation (21), and bacteria may possess more than one mechanism. The identification of the specific gene mutation(s) resulting in kanamycin resistance in mutants obtained in both the presence and absence of tea tree oil would allow the identification of an absent mutant subset.

The effects of tea tree oil or terpinen-4-ol on the development of multistep antibiotic resistance were minimal when evaluated by the standard MIC assessment criteria, whereby differences in the MIC of one doubling dilution are not considered to be significant (2, 7). However, using statistical analyses, significant differences were evident between treatments on some days. In the majority of instances, MICs were significantly lower when tea tree oil or terpinen-4-ol was present, and significant differences occurred mostly on days 1 and 2. This indicates synergistic antimicrobial interactions rather than a true alteration in resistance. It also remains possible that some of the changes in antibiotic susceptibility were the result of phenotypic adaptation rather than true resistance. Similarly to the single-step studies, the combination of tea tree oil and kanamycin appears to have influenced the development of multistep resistance in *E. coli*; however, testing with additional isolates is required to confirm this. Overall, since the presence of tea tree oil or terpinen-4-ol resulted in only minor changes in antibiotic susceptibility, and no consistent trends were apparent for either *S. aureus* or *E. coli*, it is reasonable to conclude from these data that tea tree oil and terpinen-4ol do not have a significant impact on the development of multistep antibiotic resistance.

The repeated exposure of *S. aureus* and *S. epidermidis* strains to terpinen-4-ol did not induce significant changes in antimicrobial susceptibility, which is largely in agreement with previously published data indicating minor changes in susceptibility (of 2-fold or less) after exposure to tea tree oil for similar Gram-positive organisms (23, 24). Furthermore, where changes of 4-fold or more occurred, susceptibility was largely increased rather than decreased. These data suggest that if adaptive measures were induced by terpinen-4-ol or tea tree oil, they were not sufficient to alter antimicrobial susceptibility or confer cross-protection to other antimicrobial agents.

Of the few previous studies that have attempted to induce resistance to essential oils or components, most have found either minor decreases in susceptibility or no change (1, 13, 24, 25, 28). This is similar to the present study, where minor susceptibility changes were seen by microdilution but not by macrodilution. Precisely why changes in susceptibility were observed by one method and not the other remains to be determined. Minor changes in essential oil susceptibility most likely are explained by phenotypic adaptation, which confers a low level of tolerance and has been shown to occur via reversible changes in membrane lipid composition (10, 31) and efflux (26). Organisms expressing the multiple antibiotic resistance (Mar) phenotype also have moderately reduced tea tree oil susceptibility (18). Given that many essential oil components, including monoterpenes, are lipophilic and target the structure, function, and integrity of microbial membranes, it seems unlikely that true resistance will arise.

In conclusion, this study found that exposure to tea tree oil did not have any global effects on the development of antibiotic resistance in the tested strains of *S. aureus*, *S. epidermidis*, and *E. coli*. Furthermore, no decreases in antimicrobial susceptibility were observed after repeated exposure to the monoterpene terpinen-4-ol. Little evidence was found to support the concern that the increased use of tea tree oil in both domestic and health care environments will lead to increased antimicrobial resistance.

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