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Habituation to sub-lethal concentrations of tea tree oil (*Melaleuca alternifolia*) is associated with reduced susceptibility to antibiotics in human pathogens

M. Ann S. McMahon[1]*, Ian S. Blair[1], John E. Moore[2] and David A. McDowell[1]

[1]Food Microbiology Research Group, University of Ulster, Newtownabbey, Northern Ireland BT37 OQB, UK; [2]Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Northern Ireland BT9 7AD, UK

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***Objectives*: To investigate the effect of sub-lethal challenge with tea tree oil (TTO) on the antibiotic susceptibility profiles of significant human pathogens and commensals.**

***Methods*: The study compared the antibiotic susceptibility (Etest) patterns of *Escherichia coli*, *Staphylococcus aureus*/methicillin-resistant *S. aureus* (MRSA) and *Salmonella* spp. after broth culture for 72 h in the presence or absence of sub-lethal concentrations of TTO (0.25%, 0.25% and 0.1%).**

***Results*: All habituated cultures (exposed to sub-lethal concentrations of TTO) displayed reduced susceptibility to a range of clinically relevant antibiotics compared with non-habituated (control) cultures.**

***Conclusions*: Although TTO may be an effective antimicrobial agent when appropriately used at bactericidal concentrations, its application at sub-lethal concentrations may contribute to the development of antibiotic resistance in human pathogens.**

Keywords: antibiotic susceptibility profiles, MRSA, antibacterial agents

Introduction

Tea tree oil (TTO), principally extracted from *Melaleuca alternifolia*, a native to north-eastern New South Wales, has been used within Australia as a botanical medicine for hundreds of years.[1] TTO is suggested to contain more than 100 bioactive compounds, but its main antimicrobial component is terpinene-4-ol, which is thought to induce structural damage in bacterial and fungal cell walls and membranes, compromising their abilities to maintain cell integrity.[1] TTO is effective as a bactericide at concentrations of 0.003–2%,[2] and as a fungicide at concentrations of 0.004–0.25%.[3] It has been used in reducing methicillin-resistant *Staphylococcus aureus* (MRSA) colonization,[4] and in the treatment of a wide range of infections including candidiasis and bacterial vaginosis.[5]

TTO has been shown to be effective in the clinical treatment of herpes labialis (cold sores),[1] although the exact mechanism(s) of such virucidal action are as yet unknown.[6] Despite the absence of a full understanding of its bactericidal, fungicidal and virucidal mechanisms, it is increasingly widely recommended as an effective broad-spectrum antimicrobial agent, and, as such, is used extensively in pharmaceuticals, cosmetics, toiletries, pet care and household cleaning agents.[7]

Much of this usage involves preparations that contain unknown or undisclosed concentrations of the above active agent(s); thus there is considerable opportunity for them to be applied at ineffective sub-lethal conditions. Current research into the antimicrobial activity of TTO has focused on its use in (high) bacteri-, fungi- or viru-cidal concentrations. However, less attention has been paid to the wider implications of its application in inappropriate or sub-lethal concentrations.

The aim of this study was to investigate the effect of sub-lethal challenge with TTO on the antibiotic resistance profiles of significant human pathogens and commensals.

Materials and methods

Determination of sub-MIC of tea tree oil against test organisms

The bacterial strains used in this study included a range of frequently isolated human pathogens and commensals: *Escherichia coli* ATCC 33694 and ATCC 35695; *Salmonella enterica* serovar Enteritidis NCTC 12694; *S. enterica* serovar Typhimurium DT104 St11 and St17 (Food Microbiology Research Group UUJ); *S. aureus* NCTC 8325; and MRSA 651 and 770 (Food Microbiology Research Group

*Corresponding author. Tel: +44-2890-368659; Fax: +44-2890-368419; E-mail: ma.mcmahon@ulster.ac.uk

UUJ). Each strain was grown in Luria–Bertani broth (LBB; Merck) at 37°C for 17 h, harvested by centrifugation (8000 **g** for 10 min), washed twice in sterile phosphate-buffered saline (PBS; pH 7.4), and resuspended in PBS to form standard cell suspensions with OD_{600} values of 0.1 ($\sim 10^7$ cfu/mL).

TTO (Holland & Barrett; pharmaceutical grade: <5% cineole, >35% terpinen-4-ol) was solubilized with polyoxyl 35 castor oil (Fluka; 15%),[7] and diluted in sterile distilled water to produce a 10% (v/v) stock solution which was stored at 4°C for a maximum of 5 days before use. Serial 2-fold dilutions of TTO stock solution were prepared in LBB in sterile 96-well microdilution plates (Sarstedt). LBB was added to the first well in each row of each plate to provide sterility and no growth (plate reader) controls.

Each standardized cell suspension (10 μL) was inoculated into three TTO dilution series. All plates were incubated in a humidified chamber at 37°C for 24 h and examined for cell growth as estimated by measurement of absorbance at 660 nm using a Titertek Plus Plate Reader (ICN Biomedicals Ltd). In each case the MIC was determined as the lowest concentration of TTO at which the OD reading was <0.01 at 660 nm. The concentration of TTO in the last well showing growth was defined as the (maximal) sub-lethal concentration to be used in the subsequent studies.

TTO habituation of bacterial strains

LBB (10 mL, control samples) and LBB supplemented with TTO to sub-lethal concentrations (10 mL, LBB_{TTO}) were inoculated with 100 μL volumes of the standardized cell suspensions, incubated at 37°C, and subcultured daily for 3 days into fresh LBB or LBB_{TTO} as appropriate.

Cells were recovered from LBB (control) and LBB_{TTO} (habituated) cultures by centrifugation as above, washed in PBS, and diluted to form standardized inocula equivalent to a 0.5 McFarland standard, for subsequent examination by Etest.

Determination of antibiotic susceptibility profiles for habituated (and control) cell suspensions

Antibiotic susceptibility profiles were determined by the BSAC reference method[8] using Etest strips (Biostat Ltd, Stockport, UK). MICs were determined using the manufacturers recommended method. The antibiotic susceptibility profile for each strain was determined on three occasions.

Results

The MICs of TTO required to inhibit the growth of the test organisms were determined as: *E. coli*, 0.5%; *S. aureus*, 0.5%; and *Salmonella* spp., 0.25%, allowing the derivation of sub-lethal concentrations of TTO to be used in the study as: *E. coli*, 0.25%; *S. aureus*, 0.25%; and *Salmonella* spp, 0.1%.

The antibiotic susceptibility profiles of non-TTO treated (control) and TTO habituated test organisms are shown in Table 1. MIC values for TTO habituated *E. coli* ATCC 33694 were increased for 8/10 of the antibiotics used, with >4-fold increases in MIC for 6 antibiotics. This organism is constitutively resistant to streptomycin and fusidic acid. Increased MIC values were also recorded for *E. coli* ATCC 35695 for 6/10 antibiotics used, with >2-fold increases for three antibiotics (this organism is constitutively resistant to streptomycin, vancomycin and fusidic acid).

TTO habituated *Salmonella* Typhimurium St11 and *Salmonella* Enteritidis NCTC 12694 had increased MIC values (2-fold or greater) for 5/10 antibiotics tested. TTO habituated *Salmonella* Typhimurium St17 had increased MIC values (>2-fold) for 2/10 of the antibiotics tested. All *Salmonella* strains were constitutively resistant to vancomycin and fusidic acid. In addition both *Salmonella* Typhimurium isolates were constitutively resistant to ampicillin.

Table 1. Antibiotic resistance profiles for control (non-TTO treated) and TTO habituated bacterial strains

		MIC (mg/L)									
Bacterial strain	Treatment	GEN	ERY	STR	VAN	CHL	TET	TMP	AMP	FUS	MUP
E. coli ATCC 33694	control	0.25	6	>256	64	1.5	0.25	0.016	1	>256	128
	TTO treated	0.75	24	>256	>256	8	1.5	0.25	2	>256	>1024
E. coli ATCC 35695	control	0.094	8	>256	>256	3	0.5	0.125	1.5	256	48
	TTO treated	0.19	24	>256	>256	8	0.75	0.25	1.5	>256	384
Salmonella Enteritidis	control	0.5	32	2	>256	4	1	0.19	0.75	>256	64
NCTC 12694	TTO treated	1	48	2	>256	8	3	0.38	1	>256	>1024
Salmonella	control	0.75	24	32	>256	96	24	0.25	>256	>256	128
Typhimurium St11	TTO treated	1	32	64	>256	>256	48	0.5	>256	>256	>1024
Salmonella	control	1	32	32	>256	192	32	0.38	>256	>256	128
Typhimurium St17	TTO treated	0.5	32	48	>256	>256	48	0.25	>256	>256	>1024
S. aureus NCTC 8325	control	0.19	0.19	2	2	3	0.047	0.094	0.064	0.032	<0.064
	TTO treated	0.5	0.19	1.5	2	6	0.25	0.25	0.064	0.064	0.125
MRSA 770	control	24	>256	>256	0.75	1	0.19	0.38	32	0.032	4
	TTO treated	32	>256	>256	3	6	0.38	0.75	96	0.064	24
MRSA 651	control	24	>256	>256	1	1	0.19	0.38	32	0.032	6
	TTO treated	32	>256	>256	2	12	0.38	0.75	48	0.094	32

GEN, gentamicin; VAN, vancomycin; CHL, chloramphenicol; ERY, erythromycin; TET, tetracycline; STR, streptomycin; TMP, trimethoprim; AMP, ampicillin; FUS, fusidic acid; MUP, mupirocin.

TTO habituated *S. aureus* NCTC 8325 had increased MIC values for 6/10 antibiotics tested.

TTO habituated MRSA had increased (2-fold or greater) MIC values for 7/10 antibiotics tested (MRSA are constitutively resistant to streptomycin and erythromycin).

Discussion

The range of available forms and uses of TTO have increased dramatically over the past 10 years. In addition to the pure oil format, TTO is now retailed in numerous product formats for personal health care, home care and pet care.[7] Although predominantly used by the general public, TTO is being used in many institutions such as hospitals and nursing homes, frequently as a complementary/alternative medicine. These patterns of usage mean that TTO is being used against a diverse range of human commensals and pathogens originating from kitchen/bathroom/human and animal sources. Thus a wide range of organisms of significance in public health are coming into contact with sub-lethal concentrations of TTO.

This study confirms that TTO is an effective antibacterial agent even at relatively low concentrations (0.25–0.5%), which are in line with values reported previously.[1] This study observed that 72 h habituation of all species examined (*E. coli*, *S. aureus*/MRSA, *Salmonella* Typhimurium and *Salmonella* Enteritidis) to sub-lethal concentrations of TTO was associated with reductions in antibiotic susceptibility. Increased MICs were recorded for all organisms against the majority of the 10 antibiotics tested. For example, TTO habituated *E. coli* showed >4-fold increases in MIC for 6/10 of the antibiotics tested. Habituated MRSA showed 2-fold or greater increases in MIC for 7/10 of the antibiotics tested including clinically significant increases in MICs for chloramphenicol (MRSA 651) and trimethoprim (MRSA 651 and 770),[8] as well as increases in MIC for mupirocin, the topical antibiotic of choice for the treatment/decontamination of MRSA patients.[4]

The recommended concentration for use of TTO as an antimicrobial agent is 4–5%.[4] For example, a combination of 4% TTO nasal ointment and 5% TTO body wash has been reported as an effective alternative method for the decolonization of MRSA patients.[4] However, the hydrophobic nature of pure TTO oil may make it difficult to achieve accurate direct dilution of the oil in water, thus the in-use concentration of TTO may be very different from the concentrations recommended above. Further concerns are related to reports that many products described as 'containing tea tree oil' may contain significantly lower concentrations than that recommended, and the absence of legislation regarding the minimum concentration of TTO presented in such products (B. McGilvray, personal communication). Thus there are considerable risks that TTO is being (unwittingly) used at inappropriately low (sub-lethal) concentrations.

The decrease in antibiotic susceptibility reported in this study may be attributable to: (i) the selection of TTO-resistant sub-populations during habituation—such stress hardened[9] sub-populations have been shown previously to have increased resistance to antibiotics;[10–13] (ii) phenotypic alterations within the TTO habituated bacterial cells, e.g. up-regulation of the *mar* efflux pump,[5,11] or (iii) genotypic alterations such as chromosomal mutations or acquisition of plasmid-mediated resistance (the mechanisms mentioned and their implications are extensively reviewed by Gilbert and McBain).[14]

Conclusion

Although TTO is an effective antimicrobial agent when correctly used at bactericidal concentrations, the results of this study suggest its use at sub-lethal concentrations can lead to the development of antibiotic resistance in human pathogens and commensals.

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Transparency declarations

None to declare.

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