Mechanism of action of naturally occurring antimicrobials against *Clostridium difficile*

Niloufar Roshan[a], Thomas V. Riley[a-d], Daniel R. Knight[c], James H. Steer[a] and Katherine A. Hammer[a]

[a] School of Biomedical Sciences, The University of Western Australia, Western Australia.
[b] PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Western Australia.
[c] School of Veterinary & Life Sciences, Murdoch University, Western Australia.
[d] School of Medical & Health Sciences, Edith Cowan University, Western Australia.

INTRODUCTION

- *Clostridium difficile* causes infections of the gastrointestinal tract.[1]
- Disease severity can range from self-limiting diarrhea to severe manifestations such as pseudomembranous colitis and toxic megacolon. [2]

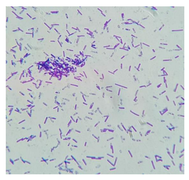


Figure 1. Gram stain of *C. difficile*

- *C. difficile* infection (CDI) has been managed with the conventional antimicrobials metronidazole (for mild to moderate CDI) and vancomycin (for severe CDI). [3]
- Several issues are associated with the use of conventional agents, including a high recurrence rate of 20-25% and reduced *in vitro* efficacy. [3]
- Natural compounds generally have broad-spectrum antimicrobial activity with several showing activity against *C. difficile.* [4]
- Compounds derived from plant extracts have great antimicrobial potential against drug-resistant microorganisms and, unlike conventional antimicrobials, they are less susceptible to the development of antimicrobial resistance . [4,5]

OBJECTIVE

This study aimed to investigate the mechanism of action of natural occurring compounds and plant extracts against *C. difficile*.

METHODS

The mechanism of action of five compounds with bactericidal activity (cinnamon root powder, peppermint oil, *trans*-cinnamaldehyde, menthol and zingerone), and four with bacteriostatic activity (fresh garlic bulb extract, garlic clove powder, *Leptospermum* honey and allicin) against two *C. difficile* strains was investigated.

Assays:

1. Bactericidal activity: Time kill kinetics and ATP-leakage assays.
2. Alterations in cell permeability: determination of protein leakage using the Bradford assay and propidium iodide uptake assays.
3. Inhibitory effect of compounds on prokaryotic translation/protein synthesis: An *in vitro* transcription/translation experiment using Promega's *E. coli* S30 extract system for circular DNA linked to the Steady-Glo®.

Figure 2. Time-kill assay viable counts

Figure 3. Prokaryotic translation/protein synthesis

4. Detection of antimicrobial cross-resistance: broth microdilution, comparing the MICs of products against a panel of antimicrobial *C. difficile* strains previously characterised for AMR phenotype and associated genotype.

RESULTS

Time-kill kinetics

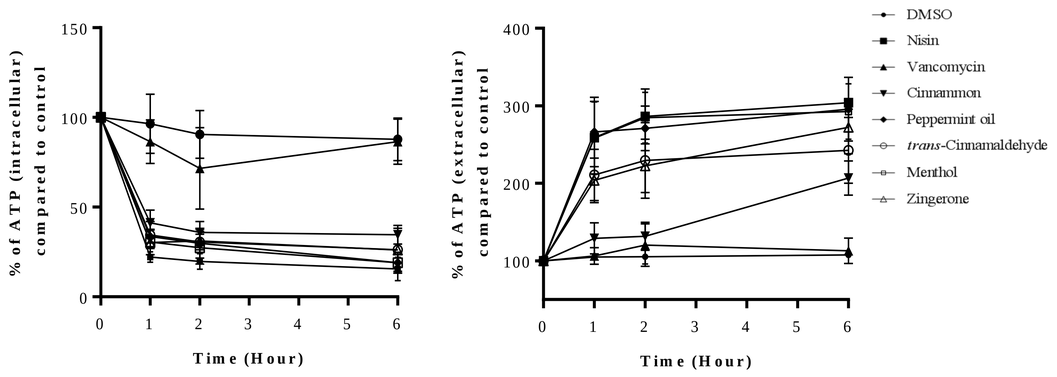
Table 1. Mean log10 cfu/ml reductions for *C. difficile* NCTC 13366 following of exposure to treatments.

Treatment	Conc	*Clostridium difficile* NCTC 13366									
		1h		2h		4h		6h		24h	
		EXP	STA	EXP	STA	EXP	STA	EXP	STA	EXP	STA
Cinnamon	2 × MIC	0.7 ± 0.3	1.6 ± 0.4	1.4 ± 0.2	2.4 ± 0.3	1.5 ± 0.2	2.6 ± 0.2	1.8 ± 0.5	2.8 ± 0.2	2.2 ± 0.6	3.6 ± 0.4
	4 × MIC	2.7 ± 0.3	NA	2.7 ± 0.4	NA	3.2 ± 0.7	NA	3.6 ± 0.5	NA	3.7 ± 0.4	NA
	8 × MIC	2.8 ± 0.4	NA	3.0 ± 0.7	NA	3.5 ± 0.6	NA	3.6 ± 0.5	NA	3.8 ± 0.4	NA
Peppermint oil	2 × MIC	2.0 ± 0.2	2.1 ± 0.5	2.2 ± 0.2	2.2 ± 0.5	2.6 ± 0.4	2.8 ± 0.3	3.1 ± 0.2	3.1 ± 0.3	3.6 ± 0.4	3.8 ± 0.7
	4 × MIC	2.5 ± 0.4	2.1 ± 0.4	3.0 ± 0.4	2.3 ± 0.5	3.0 ± 0.4	2.9 ± 0.3	3.6 ± 0.2	3.2 ± 0.3	3.9 ± 0.1	3.8 ± 0.6
	8 × MIC	2.5 ± 0.4	NA	3.1 ± 0.4	NA	3.2 ± 0.3	NA	3.7 ± 0.2	NA	3.9 ± 0.2	NA
trans-Cinnamaldehyde	2 × MIC	2.6 ± 0.8	1.1 ± 0.3	3.1 ± 0.5	1.7 ± 0.4	3.6 ± 0.6	2.0 ± 0.4	4.1 ± 0.2	2.6 ± 0.2	4.2 ± 0.2	3.0 ± 0.2
	4 × MIC	2.7 ± 0.6	1.2 ± 0.3	3.2 ± 0.2	2.3 ± 0.5	3.6 ± 0.7	2.8 ± 0.3	4.2 ± 0.2	3.1 ± 0.1	4.2 ± 0.1	3.3 ± 0.4
	8 × MIC	2.9 ± 0.6	1.7 ± 0.4	3.6 ± 0.4	2.8 ± 0.3	3.8 ± 0.7	3.2 ± 0.3	3.7 ± 0.6	3.3 ± 0.2	4.2 ± 0.2	3.3 ± 0.3
Menthol	2 × MIC	0.4 ± 0.4	1.8 ± 0.5	1.2 ± 0.3	2.2 ± 0.2	1.5 ± 0.5	2.5 ± 0.1	2.6 ± 0.9	2.5 ± 0.1	3.1 ± 0.9	3.6 ± 0.2
	4 × MIC	1.6 ± 0.7	2.2 ± 0.9	1.8 ± 0.8	2.5 ± 0.7	3.9 ± 0.2	2.9 ± 0.4	3.9 ± 0.2	3.1 ± 0.3	4.0 ± 0.1	3.7 ± 0.2
	8 × MIC	3.2 ± 0.5	2.2 ± 0.9	3.5 ± 0.6	2.7 ± 0.9	4.0 ± 0	3.2 ± 0.5	4.0 ± 0	3.2 ± 0.4	4.1 ± 0.2	3.9 ± 0.1
Zingerone	2 × MIC	2.1 ± 0.6	1.4 ± 0.6	2.9 ± 0.5	2.0 ± 0.6	2.9 ± 0.6	2.5 ± 0.4	2.9 ± 0.6	2.6 ± 0.2	3.0 ± 0.6	3.0 ± 0.1
	4 × MIC	2.5 ± 0.4	2.3 ± 0.4	3.1 ± 0.4	2.6 ± 0.1	3.3 ± 0.3	2.8 ± 0.1	3.4 ± 0.4	3.0 ± 0.2	4.2 ± 0.7	3.2 ± 0.4
	8 × MIC	3.1 ± 0.2	2.4 ± 0.6	3.4 ± 0.1	2.8 ± 0.3	3.7 ± 0.3	3.0 ± 0.3	4.3 ± 0.6	3.2 ± 0.2	4.2 ± 0.2	3.3 ± 0.5

EXP, exponential; STA, stationary; MIC, minimum inhibitory concentration; Conc: concentration; NA, not applicable;.
Sterile distilled water (SDW) and dimethyl sulfoxide (DMSO) (2.5% V/V) were used as negative controls.

The time-kill assay showed a > 3 log_{10} reduction in *C. difficile* counts by all five bactericidal compounds after 24 h. Peppermint oil at ≥ 1 × MIC resulted in a log_{10} reduction of ≥ 3 against both log- and stationary-phase *C. difficile* after 24 h. A similar pattern of killing with a reduction in bacterial counts was observed with *trans*-cinnamaldehyde at almost all concentrations.

ATP-leakage assay



The ATP-leakage assay showed that all five bactericidal compounds at most concentrations significantly reduced the intracellular ATP after 1 h of incubation ($P \leq 0.01$). The extracellular ATP was increased significantly by all five antimicrobials at all concentrations after 2 h ($P < 0.001$).

Figure 2. Effect (mean ± SD) of five bactericidal products and comparators in the leakage of ATP from the *C. difficile* cells, strain NCTC 13366. Concentration of treatments used in this assay: Nisin 8 × MIC (4 µg/ml), vancomycin 8 × MIC (8 µg/ml), cinnamon root powder 8 × MIC (150 mg/ml), peppermint oil 8 × MIC (16% v/v), *trans*-cinnamaldehyde 8 × MIC (1.6% v/v), menthol 8 × MIC (18.8 mg/ml), zingerone 8 × MIC (37.5 mg/ml).

Cell permeability assays

Table 2. Effect of products on *C. difficile* NCTC 13366, measured by protein leakage and propidium iodide uptake assays.

Treatment	Protein leakage assay			Propidium iodide uptake		
	1h	2h	6h	1h	2h	6h
Cinnamon root powder						
1 × MIC (75 mg/ml)	7.0 ± 2.3	3.0 ± 1.8	24.8 ± 5.7	324 ± 53	376 ± 136	**602 ± 128**
2 × MIC (150 mg/ml)	4.7 ± 5.0	24.4 ± 10.6	**83.1 ± 10.6**	446 ± 64	**616 ± 168**	**1061 ± 228**
Peppermint oil						
1 × MIC (8% v/v)	19.2 ± 6.8	23.6 ± 5.2	**74.3 ± 3.9**	241 ± 60	444 ± 108	**1206 ± 186**
2 × MIC (16% v/v)	**29.6 ± 9.1**	**62.9 ± 2.7**	**90.7 ± 11.2**	431 ± 156	548 ± 111	**1387 ± 294**
4 × MIC (32% v/v)	**32.8 ± 11.0**	**74.1 ± 2.6**	**99.5 ± 8.7**	256 ± 14	399 ± 142	**1061 ± 228**
trans-Cinnamaldehyde						
1 × MIC (0.02% v/v)	0.0 ± 0.0	0.2 ± 0.3	2.2 ± 3.8	**548 ± 156**	**854 ± 181**	**523 ± 10.8**
2 × MIC (0.04% v/v)	0.0 ± 0.0	3.9 ± 3.9	6.9 ± 5.9	**724 ± 43**	**1831 ± 69**	**745 ± 14.5**
4 × MIC (0.08% v/v)	0.0 ± 0.0	9.1 ± 7.4	18.2 ± 8.1	**985 ± 22**	**1186 ± 37**	**618 ± 8.1**
8 × MIC (0.16% v/v)	**27.2 ± 14.9**	**31.0 ± 21.3**	**45.9 ± 12.7**	**1115 ± 46**	**1446 ± 70**	**1444 ± 49**
Menthol						
1 × MIC (9.4 mg/ml)	0.0 ± 0.0	36.0 ± 6.9	**42.2 ± 17.6**	308 ± 12	315 ± 99	223 ± 21
2 × MIC (18.8 mg/ml)	**24.6 ± 14.8**	**37.2 ± 12.6**	**51.1 ± 13.3**	287 ± 21	287 ± 88	**487 ± 99**
4 × MIC (37.5 mg/ml)	**37.7 ± 4.8**	**51.5 ± 16.7**	**62.3 ± 22.6**	337 ± 8.9	**463 ± 101**	**513 ± 88**
8 × MIC (75 mg/ml)	**24.2 ± 8.9**	**48.8 ± 20.1**	**76.6 ± 13.5**	**461 ± 84**	**499 ± 88**	**987 ± 44**
Zingerone						
1 × MIC (9.4 mg/ml)	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	**698 ± 73**	**595 ± 20**	**990 ± 99**
2 × MIC (18.8 mg/ml)	11.5 ± 5.4	5.2 ± 3.3	3.1 ± 3.3	**739 ± 67**	**574 ± 19**	**1247 ± 29**
4 × MIC (37.5 mg/ml)	5.7 ± 3.3	20.3 ± 9.6	35.2 ± 15.3	**1263 ± 425**	**919 ± 21**	**1301 ± 12**
8 × MIC (75 mg/ml)	**38.9 ± 13.9**	**50.7 ± 13.4**	**41.7 ± 15.1**	**1101 ± 418**	**1601 ± 122**	**1214 ± 57**

MIC, minimum inhibitory concentration; Values in bold differ significantly from controls (SDW and DMSO).
Sterile distilled water (SDW) and dimethyl sulfoxide (DMSO) (2.5% V/V) were used as negative controls.
Values in bold differ significantly from controls (SDW and DMSO).

All five bactericidal compounds damaged the cell membrane in both cell permeability assays. Treatment with peppermint oil, *trans*-cinnamaldehyde, menthol and zingerone resulted in significant increases in OD_{595} indicating protein leakage after 1 h of exposure compared to the untreated controls (SDW and DMSO) ($P \leq 0.04$). Treatment with all natural compounds at most tested concentrations resulted in a significant increase in propidium iodide fluorescence after 6 h of exposure ($P < 0.05$).

Detection of antimicrobial cross-resistance

Table 3. MIC values for products against antimicrobial resistant and susceptible *C. difficile.*

Isolate ID	Phenotype	Treatment								
		Fresh garlic extract (% v/v)	Garlic clove powder (mg/ml)	Allicin (mg/ml)	*Leptospermum* honey (MGO 514+) (% w/v)	Peppermint oil (% v/v)	Cinnamon root powder (mg/ml)	Menthol (mg/ml)	Zingerone (mg/ml)	*trans*-cinnamaldehyde (% v/v)
ESP123	MLS_B/tetR	0.4	9.4	9.4	8	4	75	4.7	9.4	0.02
SQ383		0.8	9.4	9.4	8	8	75	9.4	9.4	0.03
SQ357	MLS_B	0.8	9.4	9.4	16	8	75	9.4	9.4	0.01
SQ463		0.8	9.4	9.4	16	8	75	9.4	9.4	0.02
SAP019	MLS_B/tetR	0.8	4.7	9.4	8	8	75	9.4	9.4	0.03
SQ317		0.8	9.4	9.4	16	8	75	9.4	9.4	0.03
ES0474	FQR/tetR	0.8	9.4	9.4	16	8	75	9.4	9.4	0.03
ES1046		0.8	9.4	9.4	16	8	75	9.4	9.4	0.03

R, resistant; S, susceptible; MLS_B, macrolide-lincosamide-streptogramin-B; tetR, tetracycline resistant; FQR, fluoroquinolone resistant.

Inhibition of protein synthesis

Streptomycin sulfate and tetracycline were used as positive controls and both inhibited protein synthesis/prokaryotic translation. Other than streptomycin and tetracycline, *Leptospermum* honey (MGO 514+) was the only treatment that showed a reduction in RFU ratio compared to untreated controls (SDW and DMSO) with values ranging from 42.1% to 68.9%.

None of the compounds showed elevated MICs against antibiotic-resistant strains of *C. difficile* harbouring DNA gyrase mutations, or conjugative transposons carrying *ermB* and *tetM*, suggesting that antibiotic resistance mechanisms are not cross-protective for natural products.

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CONCLUSIONS

Overall, this study provides a fundamental framework regarding the possible mechanism of action of natural occurring antimicrobials against *C. difficile*. The findings indicate that damage to the cytoplasmic membrane may contribute to the mechanism of action of several naturally occurring antimicrobials against *C. difficile*. Also, a lack of cross-over mechanisms of resistance between standard antibiotics and natural compounds are shown. Further studies are required to determine the efficacy of these compounds *in vivo.*