Non-conventional antimicrobial and alternative therapies for the treatment of *Clostridium difficile* infection

Niloufar Roshan Hesari

BSc, MInfDis

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THESIS DECLARATION

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ABSTRACT

Clostridium difficile is an important cause of infectious diarrhoea, associated with the production of highly resistant spores and the acquisition of antimicrobial resistance genes. *C. difficile* infection (CDI) is commonly recognized as a nosocomial infection affecting hospitalized patients but CDI is increasingly identified in patients in the community with no hospital contact. Antimicrobial exposure remains the main risk factor for CDI as this compromises the gut microbiota although, paradoxically, antimicrobials such as metronidazole and vancomycin are the most common treatments for CDI. The increasing frequency of highly virulent *C. difficile* strains, antimicrobial treatment failures, patients with severe complications and cases with multiple recurrences have driven the search for new therapies for CDI.

Several novel and popular complementary and alternative medicines (CAMs) are used for the treatment of diarrhoeal diseases caused by agents other than *C. difficile*, and these might also be appropriate for treating CDI. When conventional therapeutic agents show limited success, patients may turn to CAMs. Alternative treatments such as herbal remedies are often less popular with conventional medical practitioners and pharmaceutical professions. However, the demand for non-conventional antimicrobial therapies is on the rise, and further studies in the field of CAM, and especially natural products, for the treatment of CDI are required. Therefore, the overarching aim of this study was to investigate the effects of natural products on *C. difficile*. The main objectives were to:

- 1) Investigate the antimicrobial activity of various natural products against *C. difficile in-vitro*.
- 2) Determine the effect of natural products on the spore cycle of *C. difficile in-vitro*.

- 3) Examine the effect of natural products on the production and activity of *C. difficile* toxins *in-vitro*.
- 4) Investigate the mechanisms of action of natural products against *C. difficile in-vitro*.
- 5) Evaluate the efficacy of products with potent *in-vitro* activity in a mouse model of CDI.

The antibacterial activity of 22 natural products was determined by agar well diffusion and broth microdilution assays against four *C. difficile* strains, three comparator organisms and four commensal gastrointestinal organisms. Fresh garlic extract had the highest activity among the raw natural products. Peppermint and the four pure compounds, *trans*-cinnamaldehyde, allicin, menthol and zingerone were the most active processed products. Among commensal flora, *Bacteroides* species showed susceptibility to most natural products that was similar to *C. difficile*, however, *Lactobacillus casei* was less susceptible. A conventional checkerboard titration method was used to determine the effect of natural products combined with vancomycin or metronidazole. Possible synergism between *trans*-cinnamaldehyde and vancomycin, and partial synergy between *trans*-cinnamaldehyde and metronidazole, was observed.

The effect of products on sporulation, spore germination and outgrowth of *C. difficile*, in addition to sporicidal activity, was examined. Effects on sporulation were determined using microscopy and a conventional spore recovery assay. Fresh onion bulb extract (6.3% v/v) and coconut oil (8.0% v/v) inhibited sporulation by all four isolates by 66-86% and 51-88%, respectively, compared to untreated controls. Fresh ginger rhizome extract (25% v/v) was also inhibitory, although to a lesser extent. A standard spore germination and outgrowth assay showed that germination was not affected by any of the products, whereas outgrowth was significantly reduced by artichoke extract (18.8 mg/ml), fresh onion bulb extract (25% v/v), *Leptospermum* honeys (8.0% w/v) and allicin (75

mg/ml) ($P < 0.01$). Sporicidal activity was investigated using a standard plate recovery assay and was found to be minimal.

Next, the effect of all products on the production and activity of toxin from four *C. difficile* strains was determined *in-vitro*. The protective effect of products against toxin activity was determined using Vero and HT-29 cells in cytotoxicity and neutral red uptake assays. The same cell lines were used to assess the indirect effect of products on toxin-mediated cytotoxicity. Furthermore, the effect of seven products on toxin production by *C. difficile* was determined using an enzyme-linked immunosorbent assay (ELISA). Zingerone (0.3 mg/ml) protected both cell lines from *C. difficile* toxin-induced cytopathic effects ($P < 0.05$) and this was confirmed by the neutral red uptake assay. Three *Leptospermum* honeys (4.0% w/v), fresh onion bulb extract (12.5% v/v) and *trans*-cinnamaldehyde (0.005% v/v) reduced toxin production and activity significantly ($P \leq 0.023$), while garlic clove powder (4.7 mg/ml) only reduced toxin activity ($P \leq 0.047$).

The mechanisms of action of products with bactericidal activity (cinnamon root powder, peppermint oil, *trans*-cinnamaldehyde, menthol and zingerone) or bacteriostatic activity (fresh garlic bulb extract, garlic clove powder, *Leptospermum* honey and allicin) was examined using two *C. difficile* strains. All bactericidal products showed a reduction in viable count of $>3 \log_{10}$ after 24 h. A significant reduction in levels of intracellular ATP was observed with these products after 1 h ($P \leq 0.01$) and damage to the cell membrane was evident, shown by the leakage of both 260nm absorbing materials and protein, and the uptake of propidium iodide. None of the products showed bacteriolysis as determined by measuring optical density at 620nm. The effect of three bacteriostatic products on protein synthesis was quantified using an *Escherichia coli* S30 extract system, with *Leptospermum* honey (8% w/v) showing significant inhibition ($P < 0.01$). Furthermore, no products showed elevated MICs against antimicrobial-resistant *C. difficile* strains. Finally, the protective effect of two pure compounds, *trans*-cinnamaldehyde and

zingerone, with potent *in-vitro* activity was examined in a mouse model of CDI. However, the results of the *in-vivo* experiments did not support the *in-vitro* findings.

In conclusion, the work presented in this thesis provides insights into the antimicrobial activity of several natural products against *C. difficile*, including their effects on the spore cycle, effects on the activity and production of toxins, as well as their possible mechanisms of action and *in-vivo* efficacy. These studies showed the antimicrobial activity of several natural products such as different garlic preparations and pure compounds including *trans*-cinnamaldehyde and menthol against *C. difficile* whilst having less effect against gastrointestinal flora. Of the 22 products, 13% showed inhibitory effects on sporulation of *C. difficile*, while 27% reduced vegetative outgrowth of *C. difficile*, which may contribute to reducing transmission and recurrence of CDI. Several products including zingerone, *Leptospermum* honeys and *trans*-cinnamaldehyde showed activity against *C. difficile* toxin, which may be an effective approach in either controlling or treating infection. Natural products showed diverse mechanisms of action with the cytoplasmic membrane being identified as a main contributing factor for most products with bactericidal activity against *C. difficile*. The *in-vivo* study did not reflect the results observed *in-vitro*. However, due to several limitations of the mouse study, the clinical efficacy cannot be ruled out. Further studies are encouraged to carry out the chemical analysis of crude extracts. Also, improved and larger clinical trials are needed to determine the efficacy of these products *in-vivo*.

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AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

This thesis contains work that has been published. In all the papers listed below, authorship was based on the 2007 Australian Code for the Responsible Conduct of Research, developed jointly by the National Health and Medical Research Council (NHMRC), the Australian Research Council (ARC) and Universities Australia.

I hereby certify that I made a significant contribution to (a) conception and design of studies, (b) analysis and interpretation of data, (b) drafting the articles and revising it critically for important intellectual content, and (d) final approval of the version to be published. For all publications on which I am the first author, I took primary responsibility for the overall management of the drafting and submission process with editorial assistance from my co-author(s).

Niloufar Roshan Hesari

24 January 2019

I, Dr. Katherine A. Hammer certify that candidate's statements regarding their contribution to each of the works listed below are correct.

Dr. Katherine A. Hammer

24 January 2019

Journal articles: Published (full text in Appendix III)

1. Thesis publications

PUBLICATION 1

Details of the work	Roshan N , Riley TV, Hammer KA Antimicrobial activity of natural products against <i>Clostridium difficile</i> in-vitro. <i>Journal of Applied Microbiology</i> 2017; 123 : 92-103.	
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Performed all experimental work, wrote the paper and revised it critically (80%).

Co-authors signatures and dates:

Thomas V. Riley

Katherine A. Hammer

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Student contribution to work

Performed all experimental work, wrote the paper and revised it critically (80%).

Co-authors signatures and dates:

Thomas V. Riley

Daniel R. Knight

Katherine A. Hammer

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Student
contribution to
work

Performed all experimental work, wrote the paper and
revised it critically (80%).

Co-authors
signatures
and dates:

Thomas V. Riley

Daniel R. Knight

James Steer

Katherine A.
Hammer

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Student
contribution to
work

Performed most of the experimental works and help in
writing the paper (50%).

Co-authors
signatures
and dates:

Connie Locher

Thomas Rippers

Katherine A. Hammer

PRESENTATIONS

1. School of Biomedical Sciences Seminar, April 2016, Perth, Australia.
Complementary and Alternative therapies in treatment of *Clostridium difficile* infection. **Roshan N**, Riley TV, Hammer KA. Oral presentation.
3. Australian Society for Microbiology Annual Scientific Meeting, July 2016, Perth, Australia. Antimicrobial activity of natural products against *Clostridium difficile* *in-vitro*. **Roshan N**, Riley TV, Hammer KA. Poster presentation.
4. Microbiology Research Working Group seminar, August 2016, Perth, Australia.
Complementary and Alternative therapies in treatment of *Clostridium difficile* infection. **Roshan N**, Riley TV, Hammer KA. Oral presentation.
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6. Global Microbiome Meeting, August 2017, Perth, Australia. Effects of natural products on several stages of the spore cycle of *Clostridium difficile* *in-vitro*. **Roshan N**, Riley TV, Hammer KA. Poster presentation.
7. PathWest Monthly Continuing Education Program Meeting, September 2017, Perth, Australia. Effects of natural products on several stages of the spore cycle of *Clostridium difficile* *in-vitro*. **Roshan N**, Riley TV, Hammer KA. Oral presentation.

8. American Society for Microbiology Annual Scientific Meeting, June 2018, Atlanta, USA. Effect of natural products on the production and activity of *Clostridium difficile* toxins *in-vitro*. **Roshan N**, Riley TV, Knight DR, Hammer KA. Poster presentation.
9. Australian Society for Microbiology Annual Scientific Meeting, July 2018, Brisbane, Australia. Effect of natural products on the production and activity of *Clostridium difficile* toxins *in-vitro*. **Roshan N**, Riley TV, Knight DR, Hammer KA. Poster presentation.
10. Australian Society for Microbiology Annual Scientific Meeting, July 2018, Brisbane, Australia. Natural products show diverse mechanisms of action against *Clostridium difficile*. **Roshan N**, Riley TV, Knight DR, Steer J, Hammer KA. Poster presentation.
11. Global Microbiome Meeting, August 2018, Perth, Australia. Effect of natural products on the production and activity of *Clostridium difficile* toxins *in-vitro*. **Roshan N**, Riley TV, Knight DR, Hammer KA. Poster presentation.
12. 6th International *Clostridium difficile* Symposium, September 2018, Bled, Slovenia. Natural products show diverse mechanisms of action against *Clostridium difficile*. **Roshan N**, Riley TV, Knight DR, Steer, J, Hammer KA. Poster presentation.
13. Marshall Center seminar, November 2018, Perth, Australia. Non-conventional antimicrobial and alternative therapies for the treatment of *Clostridium difficile* infection. **Roshan N**, Riley TV, Hammer KA. Oral presentation.

ABBREVIATIONS

°C	Degrees Celsius
µg	Microgram(s)
µl	Microliter(s)
µM	Micromoles
µm	Micrometer(s)
AEC	Animal Ethics Committee
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUG	Augmentin
BA	Blood agar
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BSA	Bovine serum albumin
CA	California
CAM	Complementary and alternative medicine
CDAD	<i>Clostridium difficile</i> associated diarrhoea
CDI	<i>Clostridium difficile</i> infection
CDT	Binary toxin
CFU	Colony forming unit
CLI	Clindamycin
CLSI	Clinical and Laboratory Standards Institute
CO	Colorado
CPE	Cytopathic effect
CRO	Ceftriaxone
CROPs	Combined repetitive oligopeptides
d	Day(s)
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency

ENA	European Nucleotide Archive
EPA	Environmental Protection Agency
ERY	Erythromycin
EXP	Exponential
FBS	Foetal bovine serum
FDA	Food and drug administration
FDX	Fidaxomicin
FIC	Fractional inhibitory concentration
FMT	Faecal microbiota transplantation
FQR	Fluoroquinolone-resistant
g	Gram(s)
GM	Growth medium
h	Hour(s)
HIS	Supplemented heart infusion agar
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel syndrome
IDSA	Infectious Diseases Society of America
IL	Illinois
kb	Kilobase(s)
kg	Kilogram(s)
L	Liter(s)
LD	Lethal dose
LH	<i>Leptospermum</i> honey
LOD	Limit of detection
LT	Heat-labile enterotoxin
MBC	Minimum bactericidal concentration
MEM	Meropenem
MET	Metronidazole
mg	Milligram(s)
MGO	Methylglyoxal
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
Min	Minute

MLS _B	Macrolide-lincosamide-streptogramin B
MM	Maintenance medium
mM	Millimoles
mm	Millimeter
ml	Milliliter
MO	Missouri
mRNA	Messenger RNA
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MXF	Moxifloxacin
NA	Not applicable
NAAT	Nucleic acid amplification test
NCTC	National Collection of Type Cultures
ND	Not done
NJ	New Jersey
NOAEL	No observed adverse effect level
NSW	New South Wales
OD	Optical density
PaLoc	Pathogenicity locus
PBS	Phosphate buffered saline
QLD	Queensland
RFU	Relative fluorescence unit
RFX	Rifaximin
RLU	Relative light unit
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Ribotype
SBA	Supplemented Brucella agar
SBB	Supplemented Brucella broth
SD	Standard deviation
SDW	Sterile distilled water
STA	Stationary
STEC	Shiga toxin-producing <i>Escherichia coli</i>
t	Time
TET	Tetracycline

tetR	Tetracycline resistant
TMP	Trimethoprim
Tn	Transposon
tRNA	Transfer RNA
TY	Tryptose yeast
TZP	Piperacillin-tazobactam
UK	United Kingdom
UPEC	Uropathogenic <i>E. coli</i>
US	United States
USA	United States of America
UV	Ultraviolet
UWA	University of Western Australia
VA	Virginia
VAN	Vancomycin
VIC	Victoria
vs	Versus
v/v	Volume/ volume
WA	Western Australia
WI	Wisconsin
w/v	Weight/ volume

Chapter 1

Literature review

The following publication has arisen from this chapter:

Roshan N, Hammer KA, Riley TV. Non-conventional antimicrobial and alternative therapies for the treatment of *Clostridium difficile* infection. *Anaerobe*. 2018; **49**: 103-11.

1.1 INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus that can cause gastrointestinal disease in humans, with clinical symptoms arising from *C. difficile* infection (CDI) ranging from mild diarrhoea to fulminant colitis ¹⁻³. There are nearly half a million cases of CDI annually in the USA, of which approximately 29,000 patients die shortly after initial diagnosis ⁴. CDI is estimated to cause over a 250,000 annual hospitalizations per year in the USA alone ⁴. An excess of more than US\$3600 is added to the healthcare cost per case of CDI, which results in a cumulative cost of more than US\$1 billion in the USA per annum ^{4,5}. Two commonly used antimicrobials for treatment of CDI are metronidazole and vancomycin; however, they also deplete the commensal gut flora and are not effective against *C. difficile* spores, resulting in a significant rate of disease recurrence ⁶⁻⁹. Fidaxomicin is another antimicrobial that is used in severe cases of CDI when the patient is not responding to vancomycin ⁶. However, it has a narrower spectrum of activity than vancomycin or metronidazole, and may thus have less effect on gut microbiota ¹⁰. Approximately 20 to 35% of patients with CDI will experience a return of symptoms or recurrence of disease after initial antimicrobial treatment; this causes substantial morbidity and puts a major burden on health care systems ¹¹.

Diarrhoea, which is the most common manifestation of CDI, can occur in a range of other gastrointestinal disorders and infections ¹. Several studies have examined the use of complementary and alternative medicine (CAM) in the treatment of such gastrointestinal disorders ¹²⁻¹⁴. These CAMs comprise practices or a group of products that are generally excluded from conventional medicine and may also be effective in the treatment of CDI ^{12,15}. The term CAM encompasses a range of practices including, but not limited to, herbal remedies, nutritional supplements, and meditation ^{14,15}. CAMs may be considered by people as a personal luxury item (for no particular health reason), as a preventative (improving health) or as a treatment option ¹⁵. Complementary and alternative therapeutic

agents that are used primarily for treating other diarrhoeal-associated diseases are worth exploring as therapeutic options for the treatment of CDI.

1.2 *C. DIFFICILE*

1.2.1 History

C. difficile was first described in 1935 by Hall and O'Toole who were working on the microbial flora of newborns' faeces ^{16,17}. The name is derived from the word Kloster meaning spindle in Greek. *C. difficile* was originally named *Bacillus difficilis*, *difficilis* meaning difficult in Latin ¹⁶. The name was given due to the difficulty in isolating and culturing this bacterium using the technologies available at the time. Later in the 1970s, the name changed to *C. difficile* and, in the late 1970s, *C. difficile* was recognized as the cause of antimicrobial-associated disease ^{17,18}. This organism was recognized as a component of the healthy neonatal flora, which produced a toxin that was only 10-100 fold less toxic than the toxin produced by *Clostridium botulinum* ¹⁸. In 1969, the introduction of the antimicrobial clindamycin into clinical practice resulted in the emergence of cases of clindamycin-associated colitis and determining the cause of this "new" entity became a subject of interest ¹⁹. During the 1980s and 1990s, the widespread and extensive use of broad-spectrum third-generation cephalosporins was recognized as the main inducer of CDI in many developed countries ¹⁹. However, it was not until the early 2000s that *C. difficile* was recognised as a serious pathogen in the healthcare system due to a dramatic increase in CDI epidemics causing significant morbidity and mortality ¹⁷.

1.2.2 Risk factors

The microbiota of the gastrointestinal tract play a major role in human health and their disruption increases host susceptibility to CDI ²⁰. Commensal flora prevent the

overgrowth of enteric pathogens such as *C. difficile* by competing for nutrients, production of bacteriocins and contributing to host responses ²⁰. Furthermore, the intestinal microbiota inhibits *C. difficile* spore germination by producing bile salt hydrolases that convert conjugated bile salts into primary bile salts such as cholate and chenodeoxycholate ^{21,22}. This phenomenon is called “colonization resistance” and is maintained by microbiota in the gastrointestinal tract ^{21,23}.

Exposure to antimicrobials causes the disruption of gut microbiota, thus increasing the risk of contracting CDI ^{20,24,25}. Prolonged duration of hospital stay, or residency in nursing homes and rehabilitation centres, are also risk factors for CDI ^{20,26}. Advanced age is another risk factor, as people over 65 years of age are at greater risk of contracting *C. difficile* ²⁷. Other risk factors for the development of CDI include immunosuppression and the use of gastric acid suppressors ²⁴.

1.2.3 Pathogenesis

There are a number of virulence factors produced by *C. difficile*, contributing to the pathogenesis of CDI. The glucosyltransferase toxins TcdA and TcdB are the main virulence factors involved in initiating disease ^{28,29}. These toxins are functionally related proteins with similar structures and are among the largest bacterial toxins known. They are encoded by the toxin genes *tcdA* and *tcdB* located on a 19.6-kb chromosomal region called the pathogenicity locus (PaLoc) (Figure 1.1) ^{30,31}. There are also three accessory genes on the PaLoc including *tcdR* (activator), *tcdC* (negative regulator) and *tcdE* (a putative holin) ^{30,32}. These accessory genes regulate the expression of *tcdA* and *tcdB* and promote toxin release ³³. Toxin A is an enterotoxin causing exudative colitis by disrupting the adherence of colonic mucosal cells to the colonic basement membrane and destroying the villous tips ³¹. Toxin B has cytotoxic activity and enters mucosal cells by endocytosis causing apoptosis ²⁸.

Figure 1.1 Organization of toxin genes. (A) Schematic representation of the pathogenicity locus (PaLoc). Toxin-encoding genes, *tcdA* and *tcdB*, are indicated in blue; regulatory genes are shown in light green (*tcdR*; positive) or red (*tcdC*; negative); and holin-encoding gene *tcdE* is shown in dark green. The direction of the arrows reflects the direction of transcription. *tcdR* positively regulates its own expression as well as the expression of *tcdA* and *tcdB* (indicated by brown arrows). *tcdC* is an anti-sigma factor that negatively regulates toxin expression by interfering with *tcdR* function. *tcdE* is involved in the secretion of toxins. (B) Schematic representation of the binary toxin locus (CdtLoc). CDT-encoding genes, *cdtA* and *cdtB*, are shown in blue. The regulatory gene *cdtR* is shown in light green. *cdtR* positively regulates the transcription of *cdtA* and *cdtB*. The figure is adapted from Chandrasekaran and Lacy ³¹.

Binary toxin (CDT) is an additional toxin produced by a smaller proportion of *C. difficile* strains such as the so-called hyper-virulent BI/NAP1/027 epidemic strains^{31,34}. CDT is comprised of an enzymatic component (CDTa) and a binding component (CDTb), which are encoded by genes *cdtA* and *cdtB* found on CDT locus. However, there are still gaps in our knowledge about this toxin and how it contributes to virulence^{29,34}. Several other putative virulence factors are produced by *C. difficile*, though, their relevance is more speculative^{4,35}. These include hydrolytic and proteolytic enzymes, such as hyaluronidase, chondroitin-4-sulphatase, heparinase, and collagenase, but their role in the pathogenesis of the disease is not well understood³⁵ although it is assumed that these enzymes may play a role in disease processes by acting against essential components of connective tissues and contributing to the tissue damage in the gut^{35,36}. In addition, evidence suggests that *C. difficile* may have an outer capsule on its cell surface that results in a strong immunogenic potential that contributes to tissue damage^{28,37}.

1.2.4 Clinical presentation of CDI

The clinical presentation of CDI ranges from mild or self-limiting diarrhoea to severe diarrhoea and sequelae such as toxic megacolon or fulminant colitis³⁸. The time between initial antimicrobial exposure and onset of symptoms may vary from 1 day to 6 weeks or in some cases even longer³⁸. The severe forms of disease, such as fulminant colitis, are the least common and usually occur in 1% to 3% of patients (Figure 1.2)³⁹. Prominent clinical symptoms include watery diarrhea that is sometimes accompanied by abdominal swelling, fever, nausea, malaise and weight loss^{27,40}. Additional symptoms such as leukocytosis and occult colonic bleeding may also occur frequently^{38,39}. In a severe case, such as fulminant colitis, severe toxicity can cause fever, diffuse abdominal pain, and distention⁴⁰.

Figure 1.2 Pathological presentation of pseudomembranous colitis.

(A) Endoscopic visualization of a sigmoid colon with pseudomembranous colitis. (B) Colon specimen obtained during a colectomy in a patient with pseudomembranous colitis; characteristics: raised, adherent yellow plaques that vary in size from 2 to 10 mm are visible on the colonic mucosa. The intervening mucosa is hyperemic but not ulcerated. The figures are taken from Bouza *et al.* ⁴⁰ and Kelly *et al.* ³⁹, respectively.

1.2.5 Laboratory diagnosis

Accurate diagnosis of CDI is important for optimal management and control of disease^{17,41}. Laboratory detection of CDI was originally based on two kinds of tests, faecal culture and toxin detection⁴². Toxin detection using a cell culture cytotoxicity assay has traditionally been regarded as the gold standard assay for the laboratory confirmation of CDI⁴²⁻⁴⁴. Alternatively, direct enzyme immunoassays are less complex and offer a shorter turn-around time, which makes them favourable over the cell culture cytotoxicity assays⁴⁵. Anaerobic stool culture is another diagnostic method with high sensitivity, but low specificity as it lacks the capacity to distinguish asymptomatic carriers from those with disease^{46,47}. Nevertheless, both culture and toxin detection are recommended for obtaining optimal results. Nowadays, molecular testing is used for the laboratory diagnosis of many infections. Nucleic acid amplification tests (NAATs) have been in use since the 1990s in the laboratory diagnosis of CDI and may be used as part of two- or three-step algorithms or as a single test in cases of documented diarrhoea^{41,44,47}. Each test has its own advantages and disadvantages and the decision to use a particular assay depends on factors such as cost and turn-around time as well as sensitivity and specificity⁴⁷.

1.3 SIMILARITIES BETWEEN *C. DIFFICILE* INFECTION AND THE OTHER DIARRHOEAL-ASSOCIATED DISEASES

CDI has a similar clinical presentation with various other gastrointestinal disorders; diarrhoea can be due to infectious and non-infectious disorders^{48,49}. For example, some of the symptoms common to patients suffering from CDI and inflammatory bowel disease (IBD) are diarrhoea, abdominal pain, and fever, and the presence of these symptoms can make it difficult to distinguish between the two diseases⁵⁰. Irritable bowel syndrome (IBS) is another gastrointestinal disorder that can produce diarrhoeal symptoms similar

to those experienced in CDI ⁵¹. The exact aetiology of IBD and IBS remains unknown, although a range of predisposing factors including exposure to specific enteric bacteria increases the risk ^{52,53}, and several studies have reported an increased risk of developing IBS and IBD post-CDI ^{54,55}. Studies have also demonstrated an increased susceptibility of patients with IBS to gastrointestinal infections including CDI ^{51,56}.

Pathogens such as Shiga toxin-producing *Escherichia coli* (STEC) are another common cause of infectious diarrhoea and share with *C. difficile* the ability to cause diarrhoea through the production of toxins ⁵⁷. Thus, toxin inactivation may be a treatment approach for infection with these two microorganisms. In addition, *Bacillus cereus* is another spore-forming pathogen that is responsible for gastrointestinal disease and the pathogenesis of disease is also associated with toxin production ⁵⁸. Approaches for inhibiting of sporulation and spore germination in *B. cereus* or inactivating its toxins are worth investigating for *C. difficile* since several similar virulence factors are shared between these pathogens.

1.4 TREATMENT OPTIONS

1.4.1 Conventional antimicrobial treatments

1.4.1.1 Metronidazole and vancomycin

For over 30 years, metronidazole and vancomycin have been the mainstay of CDI treatment ^{7,59}. Both vancomycin and metronidazole have similar cure rates in patients with initial or recurrent mild to moderate infection, however, vancomycin was reserved for severe disease due to concerns about the generation of antimicrobial-resistant organisms such as vancomycin-resistant enterococci ^{6,60}. Currently, metronidazole remains the antimicrobial of choice for mild to moderate cases of CDI based on Australian and European guidelines ^{61,62}. However according to the most recent clinical practice guideline by the Infectious Diseases Society of America (IDSA), either vancomycin or

fidaxomicin is recommended over metronidazole for an initial episode of CDI ⁶³. There are marked differences in the mechanisms of action of metronidazole and vancomycin. Metronidazole is a nitroimidazole that interferes with DNA synthesis ^{9,64}. The recommended dose of metronidazole for mild to moderate CDI is 500 mg orally three times a day for 10 to 14 days ⁶⁵. Oral vancomycin is more expensive than metronidazole, however, unlike metronidazole, it is Food and Drug Administration (FDA) approved for treating CDI ^{9,64,65}. Vancomycin is a glycopeptide antimicrobial that acts by inhibiting the second stage of cell wall synthesis in susceptible bacteria. The recommended dose for vancomycin is 125 mg orally for 10 to 14 days ⁶⁵. According to previous randomized, controlled trials, the cure rates for mild CDI with metronidazole and vancomycin were 90% and 98%, respectively ⁶⁶. In severe cases of the disease, only 76% of the patients taking metronidazole were cured, while the cure rate for those taking vancomycin was 97% ⁶⁶. Therefore, there is concern about treatment failures, as well as the increased recurrence rate with both antimicrobials (particularly metronidazole), despite their typical efficacy.

1.4.1.2 Fidaxomicin

In 2011, fidaxomicin was approved by FDA as an antimicrobial for treating CDI in adults (18 ≥ years old) ^{7,9,67}. It is a macrocyclic antimicrobial that has weak systemic absorption after oral administration and is relatively selective for *C. difficile* in comparison to other intestinal flora ^{66,67}. One important advantage of fidaxomicin over vancomycin is its bactericidal activity, whereas vancomycin has only bacteriostatic activity against *C. difficile*. Moreover, fidaxomicin has a prolonged post-antimicrobial effect while vancomycin does not ⁶⁰. A phase III trial of fidaxomicin showed that the rates of cure and recurrence in patients with the BI/NAP1/027 strain of *C. difficile* were similar to those taking vancomycin ⁶⁸. Among 64% of patients with strain types other than BI/NAP1/027,

a recurrence rate of 7.85% was observed in patients treated with fidaxomicin compared to a 25.25% recurrence rate in those treated with vancomycin ⁶⁸. However, there are still several issues to consider regarding the use of fidaxomicin. A 10-day course treatment for this antimicrobial costs 125-fold higher than either metronidazole or vancomycin for the same duration ⁶⁸ and, therefore, the high cost of fidaxomicin may restrict its use.

1.4.2 Alternative antimicrobials

1.4.2.1 Teicoplanin

Teicoplanin is a glycopeptide antimicrobial with activity against Gram-positive anaerobes such as *C. difficile* ^{1,64}. The activity of four different antimicrobials, metronidazole, vancomycin, teicoplanin and fusidic acid, against *C. difficile* was compared in a randomized, controlled clinical trial and teicoplanin was as effective as vancomycin and metronidazole for CDI ⁶⁴. However, there was a high cost associated with teicoplanin use that made it the last line drug reserved for the treatment of repeated recurrences of CDI.

1.4.2.2 Tigecycline

Tigecycline is a broad-spectrum glycylcycline antimicrobial with *in-vitro* activity against various Gram-positive and Gram-negative bacteria including several anaerobic microorganisms ^{61,64}. This antimicrobial is a novel analogue of minocycline and is used in treating skin infections, intra-abdominal infections and pneumonia ⁶¹. Previous *in-vitro* studies have reported MIC₉₀ values ranging from 0.16 to 0.25 mg/ml for all *C. difficile* isolates ^{61,69,70}. Treatment success has been observed in six cases of CDI that were refractory to both metronidazole and vancomycin ⁶¹. Another report described the successful treatment of four patients with severe CDI with tigecycline and suggested this antimicrobial as an alternative for severe refractory CDI ⁷¹. However, inhibition of gut

microbiota has been reported with this antimicrobial and more studies are required to assess the safety and efficacy of tigecycline in the treatment of CDI.

1.4.2.3 Ramoplanin

Ramoplanin is a lipoglycopeptide antimicrobial showing *in-vitro* activity against many aerobic and anaerobic Gram-positive bacteria ^{72,73}. A phase II trial compared the efficacy of ramoplanin to vancomycin in 89 patients with CDI. Ramoplanin was administered at doses of 200 or 400 mg twice daily for 10 days and the results showed efficacy equivalent to vancomycin ⁷³. However, there was not sufficient power in this study to demonstrate non-inferiority of ramoplanin.

1.4.2.4 Surotomycin

Surotomycin is a lipopeptide antimicrobial that is orally dosed; it acts by disrupting the cell membrane ⁷⁴. In a randomized, phase II trial, the effect of surotomycin in the treatment of CDI was compared with vancomycin ⁷³. The response rates with either dose of surotomycin (125 and 250 mg) were similar to that of vancomycin (92% and 87% versus 89%, respectively). Surotomycin had a lower recurrence rate compared to vancomycin (17.2% and 27.9% versus 35.6%, respectively), however, the trial was too small to show a statistically significant difference ⁷³. In addition, in the phase III trial, surotomycin did not demonstrate non-inferiority over vancomycin ⁷³.

1.4.2.5 Cadazolid

Cadazolid is an oxazolidinone-fluoroquinolone antimicrobial and acts on *C. difficile* by inhibiting protein synthesis ^{73,74}. A phase II trial showed that cadazolid treatment resulted in a better-sustained cure rate compared to vancomycin (60, 56, and 47% for 250, 500 and 1000 mg cadazolid, respectively, and 33% for vancomycin) ⁷⁴. However, there was a

low overall response rate to vancomycin. A phase III trial assessing the safety and efficacy of cadazolid was recently completed, although study results have not yet been reported (NCT01983683) ⁷⁴.

1.4.2.6 Ridinilazole

Another novel antimicrobial is ridinilazole, formerly known as SMT19969. It is a non-absorbable, narrow spectrum antimicrobial with high *in-vitro* activity against *C. difficile* ^{73,74}. In a phase II randomized clinical trial, ridinilazole was non-inferior to vancomycin with sustained clinical response rates of 67 and 42%, respectively. Also, a lower recurrence rate was observed with ridinilazole compared to vancomycin (14.3 vs. 34.8%) ⁷⁴. However, the efficacy of vancomycin in this trial was lower compared to previous trials ⁷⁴.

1.4.3 Conventional non-antimicrobial treatments

1.4.3.1 Faecal microbiota transplantation (FMT)

Faecal microbiota transplantation (FMT) or faecal bacteriotherapy is an effective method of treatment for recurrent CDI ^{67,75}, however, it is not routinely practiced in many parts of the world ⁷⁵. It can be beneficial for patients with multiple episodes of CDI due to its reduced therapeutic expenses ⁶⁷. This procedure is usually only recommended after the third recurrence of CDI, in which, transferring faeces from a healthy donor to the patient restores the normal composition of intestinal flora and stimulates the immune response in the recipient ^{67,75,76}. To overcome the hurdles concerning the lack of availability of donor faeces and safety, several stool banks have been established around the world. One of the requirements for a stool bank is its close proximity to a clinical microbiology department with experts in the field of microbiology and infectious diseases to screen and process the stool samples ⁷⁷. Moreover, the potential stool donors are derived from established blood

donors, who are previously screened with a questionnaire and interviewed for risk factors for transmissible diseases and different factors affecting the intestinal flora. The donors who pass the questionnaire phase have their faeces screened for the presence of a range of infectious microorganisms ⁷⁷.

Faecal transplants were performed for the first time in 1958 on four patients suffering from pseudomembranous enterocolitis and all four patients showed full recovery ⁷⁸. In a subsequent study published in 2015, a success rate of 92% was reported for patients with a history of multiply recurrent CDI after receiving a stool transplant ⁷⁸. In that study, 24 patients underwent the procedure and all 24 remained free of any symptoms of CDI ⁷⁸. A randomized, controlled trial by Van Nood *et al.* published in 2013 investigated the efficacy of three treatment regimens in 43 patients with recurrent CDI ⁷⁹. The patients were randomly assigned to receive an infusion of donor faeces, vancomycin or vancomycin with bowel lavage. A better resolution of the infection was observed in those treated with the infusion of donor faeces compared to the other two treatments, however the trial was halted early ⁷⁹.

Another randomized controlled trial, involving 24 patients with CDI and 20 healthy controls, assessed the effect of FMT versus vancomycin on the virome alteration in the intestine and its association with the clinical outcomes in patients with CDI ⁸⁰. Bacterial 16S rRNA and metagenomic sequencing of virus-like particles in stool samples was performed. Patients with CDI had a greater abundance of the bacteriophage *Caudovirales* and a lower *Caudovirales* diversity and richness compared to healthy controls ⁸⁰. Treatment with FMT resulted in a significant decrease in *Caudovirales*, while recovery from infection was observed in those patients where a large fraction of their enteric virome was occupied with *Caudovirales* contigs derived from FMT donors. Among patients that responded to the treatment, FMT resulted in alterations in the virome and the bacterial microbiome, while only an alternation in the bacterial community was observed

in those treated with vancomycin ⁸⁰. This suggests *Caudovirales* bacteriophages are an important factor in the therapeutic efficacy of FMT in CDI.

The success of faecal bacteriotherapy has been evident in several studies, however, the practical barriers and adverse events associated with this technique must also be considered; for instance use of colonoscopy or duodenal tubes for the administration of stool transplant causes discomfort in patients and exposes them to some risk ⁸¹. Bacteraemia, aspiration during anaesthetic, bowel perforation, flare in IBD and fever are some of the adverse events that might be experienced by patients post-faecal transplant ⁸¹. A recent study by Youngster *et al.* assessed the efficacy of orally administered frozen faecal capsules in patients with recurrent CDI ⁸², the cure rate was 91%, however, no placebo group was included ⁸².

While oral administration of faecal flora is a simple and cost-effective technique for the treatment of recurrent CDI, it also has potential limitations; capsule preparation is labour-intensive work, it requires patients' cooperation and its use is limited in patients with dysphasia ^{81,83}. Also, despite the benefits of faecal bacteriotherapy, there is uncertainty about its long-term safety ⁸¹.

1.4.3.2 Immunotherapy

Toxins are considered as prime targets for effective protection from, or treatment of, CDI since they are the main factors responsible for the symptoms observed in *C. difficile*-associated diseases. The association between the antibody responses against *C. difficile* in 63 patients with recurrent *C. difficile* associated diarrhoea (CDAD) has been previously investigated ⁸⁴. The findings showed recurrence of disease in 50% of 44 survivors. In patients with a single episode of disease, a higher serum IgG response to toxin A was detected compared to patients who had subsequent recurrent diarrhoea ⁸⁴. This illustrated the importance of an adequate immune response in providing protection against CDI

recurrences. A randomized, double-blind, placebo-controlled trial by Lowy *et al.* published in 2010 demonstrated the protective effect of monoclonal antibodies against recurrent infection in patients being primarily treated with standard antimicrobials⁸⁵. Two other randomized controlled trials involving 2655 patients with CDI who were receiving antimicrobial treatment investigated the efficacy of the monoclonal antibodies bezlotoxumab (anti-toxin B) alone or in combination with actoxumab (anti-toxin A) for prevention of recurrent CDI⁸⁶. A lower rate of recurrent CDI was observed in patients taking bezlotoxumab therapy. Although there was success of this monoclonal antibody in the treatment of CDI, there remain concerns over the costs associated with antibody production⁸⁶.

Active vaccination to prevent CDI has been one of the long-standing goals of *C. difficile* researchers. In a study by Aboudola *et al.* a high anti-toxin A antibody response was observed in 30 healthy volunteers who were given parenteral toxoid vaccine⁸⁷. This suggested the feasibility of using a vaccine to protect high-risk individuals against CDI⁸⁷. Another study showed induced protection by oral immunization with *Bacillus* spores expressing toxin A peptide repeats against lethal doses of *C. difficile* producing toxin A and B in a hamster model of CDI⁸⁸. This vaccination was based on the induction of secretory IgA with cross-reactivity against toxin B which was also heat stable⁸⁸.

The efficacy of recombinant vaccines in animal models of CDI has also been investigated in several studies⁸⁹⁻⁹⁴. In 2012, a recombinant vaccine containing attenuated chimeric *C. difficile* toxins showed induced antitoxin and protective activity against CDI in both mouse and hamster models⁸⁹. In another recent study, the effect of a tetravalent vaccine containing recombinant, attenuated TcdA, TcdB and binary toxin components CDTa and CDTb was investigated in the Syrian golden hamster model of CDI⁹⁴. The inclusion of binary toxin components significantly increased the survival rate in hamsters challenged

with BI/NAP1/027 strains compared to bivalent vaccines containing only TcdA and TcdB antigens ⁹⁴.

While there are many successful studies on vaccine efficacy, there are manufacturing challenges involved with the production of *C. difficile* toxoid vaccines. These include the safe handling of toxin, decontamination of spores and adjusting the amount of each of the toxins produced from the same strain ⁹⁰. The multinational pharmaceutical company Sanofi recently announced a decision to discontinue clinical development of its experimental toxoid vaccine. Following a planned interim analysis of a phase III clinical trial, the company released a statement, concluding that the probability of the study meeting the primary objective was low ⁹⁵.

1.4.4 Complementary and alternative therapies

Since the early 2000s, increasing incidence and recurrence rates of CDI have been observed ⁷². Also, there has been a greater emphasis on the development of alternative therapeutic approaches due to a growing failure rate for existing antimicrobial therapies. The term CAM encompasses a wide range of practices including but not limited to herbal remedies, nutritional supplements and meditation ⁹⁶. There have been investigations on a range of non-conventional therapies for the treatment of CDI, most of which are categorized as antimicrobial agents, toxin binding agents, probiotics or bacteriocins (Table 1.1).

1.4.4.1 Toxin binding agents

Toxins released by *C. difficile* initiate disease in the colonic lumen; thus, researchers have been prompted to investigate compounds that may bind to the toxins before their attachment to the enterocytes ^{28,96}. Several types of adsorbents have been investigated for the treatment of CDI including ion exchange resins such as cholestyramine and colestipol,

Table 1.1 Non-conventional complementary and alternative approaches for treatment of *C. difficile* infection.

Agents	<i>In-vitro</i> studies	Pre-clinical studies	Clinical studies
Toxin binding agents			
Colestipol/ cholestyramine	Toxin binding ⁹⁷	Therapeutic efficacy in a hamster model of clindamycin induced cecitis ⁹⁷	Several case reports of cholestyramine in treatment CDI relapses ⁷² Randomized controlled trial of colestipol in 37 patients with severe antimicrobial-associated diarrhoea ⁹⁸
Tolvamer	Toxin binding of Tolvamer ^{1,99}	Effect of tolvamer in treatment of hamsters with <i>C. difficile</i> colitis ⁹⁹ Toxin neutralizing ability of tolvamer in a rat ileal loop model ⁹⁹	Multicenter, 3-arm, randomized, double-blind controlled phase II study on 289 patient with CDAD ¹⁰⁰ Two phase III clinical trials involving more than 1100 patients with CDI ¹⁰¹
Synsorb 90	Toxin neutralizing activity ¹⁰²	Effect of synsorbs on toxin A-induced enteritis in rat ileum ^{102,103}	Phase II clinical trial on 103 patients with at least one CDI recurrence ¹⁰² Phase III trial was begun, but no data was ever published ¹⁰²
Bacteriophage	Morphological and genetic analysis of temperate phages in <i>C. difficile</i> ¹⁰⁴ Characterizing temperate bacteriophages in <i>C. difficile</i> 027 strains ¹⁰⁵ The efficacy of two phage treatments in prevention or treatment of CDI <i>in-vitro</i> ¹⁰⁶ Use of bacteriophage ΦCD27 in a human colon model system <i>in-vitro</i> ¹⁰⁷ The ability of phage combinations in effective clearance of <i>C. difficile</i> ¹⁰⁸	The potential application of phage combinations in eradication of CDI in a hamster model of acute CDI ¹⁰⁸	A pilot observational study investigating the bacteriophage transfer in 24 patients with CDI during FMT ⁸⁰
Probiotics	-	-	Double-blind, randomized, placebo-controlled study of <i>Saccharomyces boulardii</i> on 124 patients with <i>C. difficile</i> associated disease ¹⁰¹ Randomized double-blind placebo-controlled study of <i>S. boulardii</i> on 168 patients with recurrent CDI ¹⁰⁹ Prospective, placebo-controlled multicenter trial of <i>Lactobacillus plantarum</i> 299v in 21 patients with recurrent CDI ¹¹⁰

Table 1.1 (Cont.) Non-conventional complementary and alternative approaches for treatment of *C. difficile* infection.

Agents	<i>In-vitro</i> studies	Pre-clinical studies	Clinical studies
Toxin binding agents			
Bacteriocins	Antimicrobial activity of GE2270, nisin, lacticin 3147, Thuricin CD, actagardine, LFF571 and diffocins ^{111,112} Effect of nisin on spore outgrowth ¹¹³ Evaluating the activity of NVB302 for the treatment of CDI in an <i>in-vitro</i> human gut model of CDI ¹¹¹ Avidocin-CD killing activity ¹¹⁴	Efficacy of LFF571 for recurrence of CDI in hamsters ¹¹¹ Efficacy of Avidocin –CDs in Golden Syrian hamster model of acute CDI ¹¹⁴	A randomized, double-blind, placebo-controlled study on 56 healthy male and non-pregnant female subjects between 18 to 65 years of age, assessing the safety and pharmacokinetics of single and multiple oral doses of LFF571 ¹¹⁵
Natural products	Antimicrobial activity of manuka honey ^{116,117} , virgin coconut oil ¹¹⁸ , pomegranate extract ¹¹⁹ , black seed oil and myrrh ¹²⁰	-	-

polymers (e.g. tolevamer) and oligosaccharides (e.g. synsorb 90) ⁹⁶. Early studies on the ion exchange resins, colestipol and cholestyramine showed that cholestyramine was more effective at binding *C. difficile* toxins than colestipol *in-vitro* ⁹⁷. They were both initially used as adjunctive therapies with vancomycin. Later, using a hamster model, vancomycin was more effective than cholestyramine as a sole therapy. However, the co-administration of either colestipol or cholestyramine can reduce the efficacy of vancomycin ⁹⁷. The toxin-binding polymer, tolevamer is another agent that binds to *C. difficile* toxin in the intestine and, unlike ion exchange resins, does not interfere with the activity of metronidazole or vancomycin ¹. The safety and efficacy of tolevamer for mild to moderate CDI has been shown in both phases I and II trials. However, following a phase III trial, tolevamer was inferior to vancomycin and not ideal for reducing levels of *C. difficile* toxin ¹. The initial studies on oligosaccharides such as synsorb 90 revealed encouraging results with reduced recurrence rates of CDI observed in a phase II trial ¹⁰². Nevertheless, after a phase III trial, this drug was discontinued and no further data has been published ¹⁰².

1.4.4.2 Bacteriophages

Soon after the discovery of bacteriophages in 1915, research on their use for the treatment of infectious diseases began ¹²¹. There have been several bacteriophages identified that infect *C. difficile* and they all contain elements with lysogeny control such as; repressors, anti-repressors, and integrases ^{106,121}. Meader *et al.* investigated the effect of two different phage treatment regimes, one prophylactic and one remedial, in prevention and treatment of *C. difficile in-vitro* ¹⁰⁶. A specific bacteriophage (Φ CD27) induced from *C. difficile* NCTC 12712 was used in the study. A significant decrease in *C. difficile* count was reported by the remedial treatment ($P < 0.0001$), which also reduced toxin production by *C. difficile*. The prophylactic treatment prevented *C. difficile* growth ($P < 0.001$) and inhibited toxin production. Moreover, none of the phage treatment regimens affected the

gut commensal flora significantly ¹⁰⁶. In another study by Nale *et al.*, the success of phage combination therapy in reducing *C. difficile* growth was demonstrated both *in-vitro* and *in-vivo* ¹⁰⁷. Seven phages including six myoviruses and one siphovirus were studied, and single phage treatment showed an initial reduction of bacterial load, followed by the emergence of phage-resistant colonies. However, complete lysis of *C. difficile* was shown by specific phage combinations *in-vitro*. Furthermore, the oral administration of optimized phage combinations reduced *C. difficile* colonization in a hamster model of CDI ¹⁰⁷.

1.4.4.3 Probiotics

Probiotics are live microorganisms that enhance the indigenous microflora in the host and contribute to re-establishment of the normal flora following disruption ^{122,123}. The other benefits of probiotics include the stimulation of immune response and production of antimicrobial molecules that degrade bacterial toxins or block their adhesion sites ¹²⁴. *Lactobacillus* species, *Saccharomyces boulardii* and bifidobacteria are the most common microorganisms utilized as probiotics for *C. difficile* ^{25,122,123}. The severity of CDI is usually influenced by the immune response to *C. difficile* toxins; the high titres of anti-toxin A antibodies can reduce the severity of CDI. *S. boulardii* is a yeast that can upregulate the expression of anti-toxin A secretory IgA and also secrete a protease that degrades *C. difficile* toxins ^{96,122}. A double-blind placebo-controlled trial showed a significant reduction in the recurrence rate of CDI in patients taking high doses of vancomycin in combination with *S. boulardii* compared to those receiving high dose vancomycin and placebo ⁹⁶.

The use of probiotics in the treatment of other gastrointestinal disorders has also been examined. Several studies have shown the effectiveness of probiotics in the treatment of IBD ^{124,125}. The duration of diarrhoea in children with IBS was successfully reduced

following the administration of *Lactobacillus* GG¹²⁶. In another study, a mixture of probiotics, comprising three strains of bifidobacteria, four strains of lactobacilli and one strain of *Streptococcus salivarius* subsp *thermophilus*, was given to patients in remission from ulcerative colitis who were allergic or intolerant to 5-aminosalicylic (an anti-inflammatory drug used in IBS)¹²⁷. No major side effects were observed in the patients and improvement in the patients' recovery was noticed as the probiotic organisms were able to colonize the intestine¹²⁷.

1.4.4.4 Bacteriocins of probiotic organisms

Bacteriocins are another treatment option for CDI; they are ribosomally-synthesized peptides or proteins produced by different bacteria, such as some probiotic organisms¹²⁸. Bacteriocins have several unique properties which make them a viable alternative to antimicrobials. These include having either a narrow or broad spectrum of antimicrobial activity, low toxicity, the possibility of their production by probiotic organisms *in situ* and the ability to be bioengineered¹¹¹. For a bacteriocin to be considered as an alternative therapy for CDI, it needs to be as effective as currently used antimicrobials and cause only minor damage to the gut flora^{111,112}. The *in-vitro* activity of several bacteriocins against *C. difficile* has been previously demonstrated¹¹¹. Nisin and lacticin 3147 are two bacteriocins that show *in-vitro* bactericidal activity against *C. difficile* at concentrations that are comparable to vancomycin and metronidazole¹¹¹. Thuricin CD is another known bacteriocin with a two-component peptide showing a narrower spectrum of activity and subsequently making it a potential treatment for this infection¹²⁹.

Two other bacteriocins, GE2270 and its semi-synthetic derivative LFF571, have also shown antimicrobial activity against *C. difficile*¹¹¹. In a pre-clinical study using a hamster model of CDI, the survival benefits of LFF571 were compared to vancomycin at a lower dose and there were fewer recurrences¹³⁰. A randomized, double-blind, placebo-

controlled human study investigated the safety, tolerability, and pharmacokinetics of single and multiple ascending oral doses of LFF571 ¹¹⁵. Among 56 subjects, LFF571 was well-tolerated and no serious side effects were observed. A low concentration of LFF571 remained in serum with a high concentration in faeces ¹¹⁵. The result supported the future development of LFF571 as a potential treatment for CDI.

1.4.4.5 Natural products

Natural products derived from medicinal plants are a rich source of biologically active compounds ¹³¹. Of approximately 500,000 plants species found around the globe, only 1% has been investigated for the presence of novel bioactive compounds and just a small fraction of those are investigated in randomized controlled trials ¹³². Traditional and natural remedies comprise a broad group within CAM that are generally considered by many people to be less toxic than most conventional agents ^{133,134}. In many parts of the world, medicinal plants have been used as a traditional treatment for various illnesses for thousands of years ¹³³. Herbal therapies are still used as the primary source of treatment in developing countries for many diseases. For over 5000 years, traditional Chinese medicines that utilize various plant extracts, have been the main treatment among the Chinese population ^{132,134}. Among patients with gastrointestinal issues, 26% used CAM for treatment (mostly herbal/dietary therapy, acupuncture and mind/body-based approaches) with nearly half using herbal therapy ¹³. Many patients utilise herbal remedies to treat diarrhoeal diseases without having knowledge of the specific gastrointestinal problem or aetiology of the disease ^{12,13}. These herbal remedies may have a role in the treatment of CDI worthy of investigation.

1.4.4.5.1 Potent natural products/compounds

Several commercially available natural supplements and preparations such as ginger (*Zingiber officinale*), peppermint (*Mentha × piperita*) oil, and aloe vera (*Aloe vera*) are available for the relief of symptoms experienced in several gastrointestinal disorders ^{10,135}. In particular, antispasmodic and carminative properties have been shown for some essential oils ^{135,136}. Peppermint oil has been the main constituent of many remedies for treating the symptoms of IBS ¹³⁵. In a prospective, randomized, double-blind, placebo-controlled clinical study, 110 outpatients suffering from IBS were assigned to take either enteric-coated peppermint oil capsules or a placebo ¹³⁶. A significant improvement of symptoms was observed in patients taking the peppermint oil capsules compared to placebo ¹³⁶. Ginger is another natural product known for reducing nausea and vomiting ¹³⁷. In a double-blind randomized controlled pilot trial in 45 patients with IBS, ginger was well-tolerated by patients, but did not perform better than placebo in treating symptoms. Nevertheless, ginger is a common herbal medicine used by IBS patients and larger trials are needed before drawing any definitive conclusions ¹³⁷. These commonly used natural products may have potential activity against *C. difficile*.

For centuries, traditional Chinese herbal medicine has been used for the treatment of gastrointestinal disorders, particularly for functional bowel disorders such as IBS ¹³⁸. Since there is no specific conventional medical therapy for either IBD or IBS, this has directed the treatment options towards the relief of the predominant symptoms ¹². The majority of Chinese herbal formulae contain a combination of several herbal extracts with medicinal properties ¹³². The efficacy of traditional herbal Chinese medicine for the treatment of IBS has been reported in a number of studies ¹³². In a randomized, controlled trial, the effect of three Chinese herbal formulations on patients suffering from IBS was assessed, and a significant improvement in patients taking the herbal therapy was observed compared to the placebo group ¹³⁹.

Garlic and allicin

Garlic (*Allium sativum*) belonging to the Liliaceae family, is a common food spice used in many parts of the world ¹⁴⁰. Garlic as an edible plant has been used for centuries as a medicinal agent for a wide range of diseases ^{140,141}. Its antimicrobial activity has been known for a long time with a broad spectrum of activity against Gram-positive and Gram-negative bacteria ^{142,143}. Allicin is the main compound in garlic that is responsible for its antibacterial activity and its typical pungent odour ^{141,144}. Other compounds in garlic such as saponins and flavonoids may also contribute to its antibacterial activity ¹⁴⁴. Allicin is generated by crushing garlic cloves, which results in the conversion of alliin to allicin and other thiosulfinates by the alliinase enzyme ¹⁴⁴.

Peppermint oil and menthol

Peppermint (*Mentha × piperita*) is an important medicinal plant belonging to the family Lamiaceae ^{145,146}. It is usually cultivated in temperate areas of the world such as Europe, North America and North Africa, however, nowadays it is grown in most regions of the world ¹⁴⁶. The essential oils extracted from different parts of the plant have been used for the treatment or relief of symptoms in a variety of conditions such as IBS and non-ulcer dyspepsia ¹⁴⁷. The main active constituent of peppermint oil is menthol, comprising 29% to 48% of the oil ¹⁴⁵. The other active pharmaceutical constituents are menthone, cineol and several other volatile components ¹⁴⁷. Peppermint oil has a complex chemistry and is highly variable with concentrations of ingredients relatively dependent on the climate and the geographic location of growth ¹⁴⁷.

Cinnamon and trans-cinnamaldehyde

Cinnamon (*Cinnamomum cassia*), belonging to the Lauraceae family is one of the common spices used by different cultures all over the world ^{148,149}. Most parts of a

cinnamon tree including the bark, leaves, fruits, and roots have medicinal use with volatile oils derived from different parts of cinnamon having varying chemical compositions ¹⁴⁹. Cinnamon acts as a coagulant that prevents bleeding; it also exerts important activities including antimicrobial, antifungal and antioxidant effects ¹⁴⁸. Cinnamon contains an array of components, such as *trans*-cinnamaldehyde, cinnamic acid, and cinnamate ¹⁵⁰. In particular, the compound *trans*-cinnamaldehyde is a component of cinnamon bark extract that possesses a broad spectrum of activity against various pathogens ^{150,151}.

Ginger and zingerone

Ginger (*Zingiber officinale* Roscoe) belonging to the family Zingiberaceae is another common spice used throughout the world for more than 2000 years ^{152,153}. The ginger plant was originally cultivated in China and then spread to India, South East Asia, West Africa and the Caribbean ¹⁵². Ginger contains many active components that vary depending on the plant variety and geographical region where it is grown. It yields up to 3% essential oil and is commonly used for nausea, dyspepsia, and colic ^{154,155}. According to the FDA, ginger is recognized as a safe herb and in German monographs, no known side effects or drug/herb interactions were reported ¹⁵⁶. One of the major components of ginger is zingerone also known as vanillylacetone, which is a pungent pyrolytic derivative found in ginger root ¹⁵⁷. Zingerone is primarily found in dry ginger, however thermal degradation or drying also converts gingerol (another component of ginger) into zingerone. Zingerone has various pharmacological properties including antioxidant, anti-inflammatory and antimicrobial activities ¹⁵⁷.

Honey

Honey is a natural product produced by bees of the genus *Apis* using nectar from flowering vegetation. Other than being a food, honey has a long history of medicinal use

based mainly on its antimicrobial activity ^{158,159}. Several factors contribute to this antimicrobial activity including low pH, high osmolarity, the hydrogen peroxide content and phytochemical factors ¹⁶⁰. Honey is traditionally used in wound healing and as an alternative or complementary treatment for various ailments such as gastrointestinal problems ^{158,161}. Flavours and the antibacterial activity of honey vary according to the nectar source, different types and age of honey. Honeys with a *Leptospermum* floral source, in particular, manuka honey from *L. scoparium* contains the compound methylglyoxal (MGO) that contributes to the non-peroxide activity ¹⁶². *Leptospermum* honey is one of the few honeys that are highly desirable for clinical use since the non-peroxide activity is thought to provide longer lasting activity than honeys with peroxide-based activity, as the peroxide activity is quenched by catalase ^{159,163}.

1.4.4.5.2 Natural products with antimicrobial activity against *C. difficile*

A very few natural products have been evaluated for anti-*C. difficile* activity *in-vitro*. A study by Hammond and Donkor investigated the antimicrobial activity of manuka honey against three *C. difficile* strains ¹¹⁶. The MIC and MBC were 6.25% v/v for the *C. difficile* strains. The *in-vitro* bactericidal effect of manuka honey against *C. difficile* suggested this honey as a possible alternative treatment for CDI if future *in-vivo* studies showed potential ¹¹⁶. In 2017, another study by Piotrowski *et al.* investigated the effect of manuka honey against four *C. difficile* strains and on biofilm formation *in-vitro* ¹¹⁷. Similar results were shown; the manuka honey showed MIC and MBC of 6.25% v/v for all four strains. Also a significant inhibition of biofilm formation was observed ¹¹⁷. The antimicrobial effect of virgin coconut oil against *C. difficile* has also been investigated in another study ¹¹⁸. The virgin coconut oil itself did not inhibit the growth of *C. difficile*, however, lipolyzed virgin coconut oil at concentrations of 0.15% to 1.25% v/v inhibited *C. difficile* ¹¹⁸. This encourages a more in-depth evaluation of virgin coconut oil and its bioactive components

against *C. difficile*. Finegold *et al.* studied the antimicrobial activity of pomegranate extract against 29 clinical *C. difficile* isolates using an agar dilution method ¹¹⁹. Also, the phenolic contents were quantified using the Folin-Ciocalteu colorimetric method, and showed final concentrations of 6.25 to 400 µg/ml gallic acid equivalent. The extracts showed MIC values ranging from 12.5 to 25 mg/ml for all the strains, which suggested the possible efficacy of pomegranate in the treatment of CDI ¹¹⁹. Another recent study reported the antimicrobial activity of black seed oil and myrrh against two *C. difficile* strains ¹²⁰. Both black seed oil (2% v/v) and myrrh water extract inhibited *C. difficile* growth *in-vitro* as shown by an agar well diffusion assay ¹²⁰.

1.5 CONCLUSIONS

C. difficile is an important cause of disease in both humans and other animals. While standard pharmacological antimicrobial treatment is a suitable therapeutic option for most mild to moderate cases of CDI, approximately 25% of patients fail antimicrobial treatment, leading to recurrences and possible emergence of resistant strains of *C. difficile*. Evaluating CAM including a range of popular and novel compounds such as herbal extracts and essential oils that are primarily used for the treatment of other diarrhoeal diseases for therapeutic action against CDI is worthwhile. This may result in the introduction of new therapeutic options for treating CDI. Many natural products inhibit pathogens using mechanisms of action that are different from those employed by conventional antimicrobials, making it difficult for pathogens to develop resistance to natural products. Use of CAM has the potential to reduce the rate of increasing resistance to antimicrobials, as well as the costs associated with this problem.

1.6 RESEARCH AIMS

The incidence of CDI has increased internationally, particularly in the northern hemisphere. The increasing frequency of hypervirulent *C. difficile* strains, antimicrobial treatment failures, high frequency of outbreaks, patients with severe complications and cases with multiple recurrences have driven the search for alternative and new therapies. Thus, this project aimed to investigate the efficacy of a range of alternative treatments against *C. difficile*.

The specific aims of this research project were to:

1. Determine the antimicrobial activity of a range of natural products against *C. difficile in-vitro*.
2. Investigate the effect of natural products on the life cycle of *C. difficile* spores *in-vitro*.
3. Examine the effect of products on *C. difficile* toxin production and activity *in-vitro*.
4. Determine the mechanisms of action of candidate products against *C. difficile*.
5. Evaluate the efficacy of products with potent *in-vitro* activity in a mouse model of CDI.

1.7 THESIS STRUCTURE

The thesis is organized into eight chapters. Chapter One gives an introductory review, describing the current knowledge of *C. difficile*, associated diseases and the conventional and alternative therapeutic options. Chapter Two describes the materials and methods used in this study. Chapter Three describes the antimicrobial activity of 22 natural products against *C. difficile* and comparator microorganisms. This chapter also shows the effect of these products on a number of the representative commensal flora of the gastrointestinal tract. Chapter Four investigates the effect of natural products on

sporulation, spore germination and outgrowth of *C. difficile*, in addition to sporicidal activity *in-vitro*. Chapter Five examines the effect of products on *C. difficile* toxin production and activity *in-vitro*. Chapter Six investigates the mechanisms of action natural products against *C. difficile*. Chapter Seven evaluates the effect of two potential products with high *in-vitro* activity in a mouse model of CDI. Finally, Chapter Eight includes a general discussion and summarizes the major findings and conclusions of this research project.

Chapter 2

Materials and methods

2.1 MATERIALS

2.1.1 Microorganisms

Bacteria were obtained from the School of Biomedical Sciences at The University of Western Australia, and from the Department of Microbiology of PathWest Laboratory Medicine (WA). The details of these bacterial strains are provided in Table 2.1.

2.1.2 Natural products

Twenty-two natural products were selected for testing based on historical evidence, their current popularity and feasibility. The details of these products are summarized in Table 2.2. Products were categorized into three broad groups. Group 1 included raw products that people consumed as food, group 2 represented processed products that are taken as health supplements and group 3 contained the major components of some of these products. The raw products (n=10) were purchased from a supermarket in Perth, Western Australia (WA) (Table 2.2). *Leptospermum* honeys were provided by Capilano Company, Bayswater, WA. Processed and pure products (n=12) were purchased from pharmacies and health food stores in Perth, WA, or were purchased online (Table 2.2).

2.1.3 Chemicals, reagents and antimicrobials

Chemicals, reagents and antimicrobial agents are listed in Table 2.3.

2.1.4 Buffers, solutions and stains

The following buffers and solutions were prepared in distilled water and stored at room temperature, unless otherwise stated. Phosphate buffered saline (PBS) was supplied by PathWest Media, Mount Claremont, WA.

Table 2.1 Microorganisms.

Organisms	Strain/Isolate identifier	PCR Ribotype (RT)	Toxin profile
<i>Bacteroides fragilis</i> *	NCTC ² 9343	NA	
<i>Bacteroides thetaiotaomicron</i>	5018	NA	
<i>Bacteroides vulgatus</i> *	NCTC ² 10583	NA	
<i>Clostridium difficile</i> *	ATCC ¹ 700057	-	A ⁻ B ⁻ CDT ⁻
<i>Clostridium difficile</i> *	ATCC ¹ 43598	017	A ⁻ B ⁺ CDT ⁻
<i>Clostridium difficile</i> *	NCTC ¹ 13366	027	A ⁺ B ⁺ CDT ⁺
<i>Clostridium difficile</i>	R11446	014	A ⁺ B ⁺ CDT ⁻
<i>Clostridium difficile</i>	ES1046	126	A ⁺ B ⁺ CDT ⁺
<i>Clostridium difficile</i>	ES0747	126	A ⁺ B ⁺ CDT ⁺
<i>Clostridium difficile</i>	SAP019	014	A ⁺ B ⁺ CDT ⁻
<i>Clostridium difficile</i>	ESP123	014	A ⁺ B ⁺ CDT ⁻
<i>Clostridium difficile</i>	SQ317	014	A ⁺ B ⁺ CDT ⁻
<i>Clostridium difficile</i>	SQ357	014	A ⁺ B ⁺ CDT ⁻
<i>Clostridium difficile</i>	SQ383	014	A ⁺ B ⁺ CDT ⁻
<i>Clostridium difficile</i>	SQ463	014	A ⁺ B ⁺ CDT ⁻
<i>Clostridium difficile</i>	M7404	027	A ⁺ B ⁺ CDT ⁺
<i>Clostridium perfringens</i> *	ATCC ¹ 13124	NA	
<i>Escherichia coli</i> *	ATCC ¹ 25922	NA	
<i>Lactobacillus casei</i>	M2971	NA	
<i>Lactobacillus casei</i>	Q328	NA	
<i>Staphylococcus aureus</i> *	ATCC ¹ 29213	NA	

*Reference isolates; ¹ American Type Culture Collection; ² National Collection of Type Culture

NA: not applicable.

Table 2.2 Natural products investigated in this study.

Product	Concentration	Ingredient information	Supplier
<u>Raw products</u>			
Fresh garlic bulb	1 ml/ml	-	Local supermarket
Fresh onion bulb	1 ml/ml	-	Local supermarket
Fresh ginger rhizome	1 ml/ml	-	Local supermarket
<i>Leptospermum</i> honey (MGO 514+) (A)	100%	-	Barnes Naturals, Richlands, QLD, Australia
<i>Leptospermum</i> honey (MGO 263+) (B)	100%	-	Barnes Naturals
<i>Leptospermum</i> honey (MGO 263+) (C)	100%	-	Barnes Naturals
Garlic clove powder	1 mg/mg	-	Spencers, Fremantle, WA, Australia
Ginger rhizome powder	1 mg/mg	-	Spencers
Cinnamon root powder	1 mg/mg	-	Spencers
Turmeric root powder	1 mg/mg	-	Spencers
<u>Processed products</u>			
Garlic tablet	Garlic (<i>Allium sativum</i>) extract equivalent to dry bulb 10,000 mg	A	Nature's Own, Virginia, QLD, Australia
Ginger tablet	1000 mg	A	Nature's Own
Cinnamon tablet	Cinnamon (<i>Cinnamomum cassia</i>) stem bark inner powder 1000 mg	B	Swisse, Collingwood, VIC, Australia
Turmeric tablet	1000 mg	C	Nature's Way, Warriewood, NSW, Australia
Artichoke capsule	Herbal extract equivalent to <i>Cynara scolymus</i> fresh leaf 60000 mg	D	Nature's Sunshine, Baulkham hills, NSW, Australia
Coconut oil capsule	100% coconut oil	E	Nature's Way
Peppermint oil	100% pure, <i>Mentha X piperita</i> leaf 1 ml/ml	None	Oil Garden, Springvale, VIC, Australia
Aloe vera gel	99% pure <i>Aloe barbadensis</i>	F	Plunkett's, Warriewood, NSW, Australia
<u>Pure compounds</u>			
Allicin	180 mg allicin extract (garlic bulb)	G	Vcaps, Chicago, IL, USA.
<i>trans</i> -Cinnamaldehyde	Purity > 98.5%	None	Sigma Aldrich, Saint Louis, MO, USA
Menthol	Purity > 98.5%	None	Sigma Aldrich
Zingerone	Purity > 96%	None	Sigma Aldrich

MGO, methylglyoxal.

A, No added yeast, starch, gluten, lactose, sugar, artificial colours or flavours, artificial sweeteners or preservatives, dairy products or animal-derived products.

B, No added lactose, egg, gluten, soy, yeast, artificial colours and artificial flavours. C, Turmeric powder 77%, cellulose microcrystalline, povidone, silica colloidal anhydrous, magnesium stearate, croscarmellose sodium.

D, No added lactose, starch, added sugars and salt, artificial colourings and flavourings or preservatives.

E, Capsule shell (gelatin, glycerol, water).

F, Allantoin (found in Comfrey root), carbomer, disodium edetate, sodium hydroxyl methylglycinate, grapefruit seed extract

G, Vegetarian capsule (hypromellose, water), maltodextrin, gum acacia, Made without corn, wheat, gluten, yeast, soy, sugar, dairy, artificial colors or flavors

Table 2.3 Chemicals, reagents, desiccated media and antimicrobials.

Chemical, reagent or medium	Supplier
Ethanol, Absolute	Merck Pty Ltd, Darmstadt, Germany
Bacteriological agar	Astral Scientific, Taren Point, NSW, Australia
Bovine serum albumin (BSA)	Sigma Aldrich, Castle Hill, NSW, Australia
Brain heart infusion broth	Oxoid Ltd, Hants, UK
Brucella agar	Fisher Scientific, Malaga, WA, Australia
Brucella broth	Fisher Scientific, Malaga, WA, Australia
Coomassie blue G-250	Bio-Rad, Richmond, CA, USA
Dimethyl sulfoxide (DMSO)	Chem-Supply, Malaga, WA, Australia
Dulbecco's modified Eagle's medium (DMEM)	PathWest Media, Mount Claremont, WA, Australia
Fetal bovine serum (FBS)	Serana, WA, Australia
Fidaxomicin (99.9%)	Optimer Pharmaceuticals, San Diego, CA, USA
Acetic acid, glacial	Sigma Aldrich, Castle Hill, NSW, Australia
Hemin	Sigma Aldrich, Castle Hill, NSW, Australia
Horse blood	PathWest Media, Mount Claremont, WA, Australia
Hydrochloric acid (HCL)	Sigma Aldrich, Castle Hill, NSW, Australia
De Man Rogosa and Sharpe (MRS) broth	Oxoid Ltd, Hampshire, England
L-cysteine	Sigma Aldrich, Castle Hill, NSW, Australia
Malachite green (5%)	Banksia Scientific, Bulimba, QLD, Australia
Metronidazole (99.9%)	Sigma Aldrich, Castle Hill, NSW, Australia
Metronidazole disc (30 µg)	Thermo Fisher Scientific, Scoresby, VIC, Australia
Mueller-Hinton agar (MHA)	Oxoid Ltd, Hampshire, England
Neutral red	Sigma Aldrich, Castle Hill, NSW, Australia
Propidium iodide	Sigma Aldrich, Castle Hill, NSW, Australia
Safranin 0.5% aqueous solution	Amber Scientific, Midvale, WA, Australia
Sodium bicarbonate (Na ₂ CO ₃)	Merck Pty Ltd, Kilsyth, VIC, Australia
Sodium chloride (NaCl)	Merck Pty Ltd, Kilsyth, VIC, Australia
Sodium hydrogen carbonate (NaHCO ₂)	Sigma Aldrich, Castle Hill, NSW, Australia
Sodium hydroxide (NaOH)	Merck Pty Ltd, Kilsyth, VIC, Australia
Streptomycin sulfate	Sigma Aldrich, Castle Hill, NSW, Australia
Taurocholic acid	Sigma Aldrich, Castle Hill, NSW, Australia
Tetracycline	Sigma Aldrich, Castle Hill, NSW, Australia

Table 2.3 (Cont.) Chemicals, reagents, desiccated media and antimicrobials.

Chemical, reagent or medium	Supplier
Trimethoprim disc (5 µg)	Thermo Fisher Scientific, Scoresby, VIC, Australia
Triton X-100	Ajax chemicals, NSW, Australia
Trypan blue solution (0.4% g/ml)	Sigma Aldrich, Castle Hill, NSW, Australia
Trypsin	Thermo Fisher Scientific, Scoresby, VIC, Australia
Trypsin-versene concentrate	PathWest Media, Mount Claremont, WA, Australia
Tryptose	Oxoid Ltd, Hampshire, England
Ultrapure water	Fisher Biotech, Perth, WA, Australia
Vancomycin (99.9%)	Sigma Aldrich, Castle Hill, NSW, Australia
Versene solution	PathWest Media, Mount Claremont, WA, Australia
Vitamin K ₁ solution (10 mg/ml)	Sigma Aldrich, Castle Hill, NSW, Australia
Yeast extract	Oxoid Ltd, Hampshire, England

0.85% saline

NaCl	8.5 g
Distilled water	1 L

The solution was sterilized by autoclaving for 15 min at 121°C and was stored at room temperature.

Coomassie blue working solution

Coomassie brilliant blue G-250 dye	100 ml
Sterile distilled water (SDW)	400 ml

Enzyme-linked immunosorbent assay (ELISA) wash buffer

Wash buffer concentrate (20×)	50 ml
SDW	950 ml

The solution was prepared in a sterile 1 L Schott bottle.

Neutral red stock solution (4 mg/ml)

Neutral red dye	40 mg
Phosphate buffered saline (PBS) pH 7.3	10 ml

The stock solution was stored at room temperature and shielded from light.

Neutral red working solution (0.04 mg/ml)

Neutral red stock solution	0.12 ml
Dulbecco's modified's maintenance medium	11.88 ml

The working solution was incubated at 37°C with 5% carbon dioxide (CO₂) 24 h prior to use.

Neutral red destain solution

Absolute ethanol	250 ml
SDW	250 ml
Glacial acetic acid	5 ml

The reagent was prepared in a class II biosafety cabinet in a sterile Schott bottle and was stored at room temperature.

Propidium iodide stock solution (5 mM)

Propidium iodide	3 mg
PBS	1 ml

Propidium iodide working solution (5 μ M)

Propidium iodide stock solution (5 mM)	500 ml
PBS	500 ml

2.1.5 Bacterial culture media

ChromID *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) was purchased pre-prepared from bioMérieux.

The following media were manufactured and supplied by PathWest Media, Mount Claremont, WA, Australia and had been prepared according to the manufacturer's instructions:

Blood agar (BA)

Brain heart infusion agar (BHIA)

BHIA supplemented with 0.1% taurocholate and 1 mM glycine (BHIA-tag)

Brain heart infusion broth (BHIB) supplemented with 15% glycerol

BHIB (Single strength)

Brucella agar supplemented with 5 µg/ml hemin, 1 µg/ml vitamin K₁, 5% v/v laked sheep blood (SBA)

Chocolate agar

Mueller-Hinton agar (MHA)

2.1.5.1 Preparation of media and supplements

The following media and supplements were prepared according to the manufacturer's instructions and Clinical and Laboratory Standards Institute (CLSI) guidelines ¹⁶⁴.

Supplements

NaOH, 1.0 N

NaOH	8 g
Distilled water	200 ml

The solution was stored at room temperature in a tightly closed container for up to 6 months.

Hemin stock solution (5 mg/ml)

Hemin	0.1 g
1.0 NaOH	2 ml
Distilled water	18 ml

The solution was stored at 4°C, protected from light, for no longer than 1 month.

Vitamin K₁ stock solution (1 mg/ml)

Vitamin K ₁ (10 mg/ml)	0.2 ml
95% ethanol	20 ml

The solution was stored at 4°C in a dark bottle for no longer than 1 year.

Vitamin K₁ working solution (1 mg/ml)

Vitamin K ₁ stock solution	1 ml
SDW	9 ml

The solution was stored at 4°C in a dark bottle for no longer than 1 month.

Lysed horse blood (50%)

Equal volumes of defibrinated horse blood and SDW (50%) were mixed aseptically. The 50% horse blood was frozen at $\leq -20^{\circ}\text{C}$ and was freeze-thawed five to seven times until cells were thoroughly lysed. The blood mixture was clarified by centrifugation at 12000 $\times g$ for 20 min and retaining the supernatant and discarding any pelleted debris.

L-cysteine stock solution (8% w/v)

L-cysteine	2 g
SDW	25 ml

Media

All components were dissolved in distilled water and sterilized by autoclaving at 121°C for 15 min.

Double strength Mueller-Hinton broth (MHB)

Mueller-Hinton broth powder	42 g
Distilled water	1 L

Double strength supplemented BHIB

BHIB powder	37 g
Yeast extract	10 g

L-cysteine stock solution (8% v/w)	25 ml
Taurocholate	1 g
SDW	1 L

Double strength MRS broth

MRS broth powder	55 g
SDW	1 L

4 × Strength supplemented Brucella broth (SBB)

Brucella broth powder	112 g
Hemin stock solution (10 mg/ml)	4 ml
Vitamin K ₁ working solution (1 mg/ml)	4 ml
Distilled water	600 ml

The broth with hemin and vitamin K₁ was autoclaved, cooled to ≤ 48 to 50°C and 400 ml sterile lysed horse blood (50%) was added. The broth was stored at 4°C .

3% Tryptose, 2% yeast extract (TY) broth

Tryptose	30 g
Yeast extract	20 g
Distilled water	1 L

2.1.6 Tissue culture cells, media and reagents

2.1.6.1 Cell lines

Vero (African green monkey kidney) cell was provided by the Virology Section of PathWest Laboratory Medicine, Nedlands, WA, and HT-29 (human colon carcinoma)

cell was provided by Dr. Shane M. Colley from The Harry Perkins Institute of Medical Research, Nedlands, WA.

2.1.6.2 Media

Vero cells were passaged and maintained in Dulbecco's modified Eagle's medium (DMEM). HT-29 cells were passaged and maintained as described for the Vero cells, except that the medium also contained glucose (4.5 g/L). Media was supplied by PathWest Media, Mount Claremont, WA.

Supplements

DMEM was supplemented with foetal bovine serum (FBS). The 10% FBS (DMEM-GM) and 2% FBS (DMEM-MM) were used as growth and maintenance media, respectively.

Antimicrobial agents

Both DMEM-GM and DMEM-MM also contained 200 µg/ml vancomycin and 100 µg/ml streptomycin sulfate. The stock solutions were prepared in SDW, filtered through a 0.22 µm-pore size Millipore filter and stored in small aliquots at -20°C.

Vancomycin stock solution (20 mg/ml)

Vancomycin	2 g
SDW	100 ml

Vancomycin working solution (0.2 mg/ml)

Vancomycin stock solution (20 mg/ml)	1 ml
DMEM-GM or DMEM-MM	99 ml

Streptomycin sulfate stock solution (10 mg/ml)

Streptomycin sulfate	1 g
SDW	100 ml

Streptomycin sulfate working solution (0.1 mg/ml)

Streptomycin sulfate stock solution (10 mg/ml)	1 ml
DMEM-GM or DMEM-MM	99 ml

2.1.6.3 ReagentsTrypsin-versene concentrate

Trypsin	12.5 g
Versene solution	500 ml

The solution was placed on a magnetic stirrer in the 37°C warm room for 1 h and was filter sterilized using a 0.22 µm-pore size Millipore filter.

Trypsin-versene working solution (0.2% g/ml)

Versene solution	400 ml
Trypsin-versene concentrate	80 ml
NaHCO ₃ (2.8% g/ml)	10 ml
Vancomycin stock solution	4 ml
Streptomycin stock solution	4 ml

Trypsin-versene working solution was stored in small aliquots and was kept at -20°C until use. The solution was thawed at 4°C prior to use.

2.1.7 Commercial kits

The BactTitre-Glo™ microbial cell viability assay kit, *E. coli* S30 extract system for circular DNA and pBEST *luc*™ DNA kits were obtained from Promega, Madison, WI, USA. *C. difficile* TOX A/B II™ ELISA kit was supplied by TECHLAB® Blacksburg, VA, USA.

2.1.8 Other consumables

Other consumables are listed with their suppliers in Table 2.4. Disposable plasticware such as pipette tips, transfer pipettes, cryotubes and centrifuge tubes were manufactured by Nunc, Eppendorf, *In-vitro* Technologies and Samco Scientific Co.

2.2 GENERAL METHODS

2.2.1 Storage and recovery of isolates

All test organisms were stored at -20°C, in BHIB with 2% glycerol in cryovials. Prior to use, bacteria were subcultured onto BA. Anaerobic bacteria were incubated at 35°C in an A35 anaerobic chamber (Don Whitely Scientific Ltd, Shipley, West Yorkshire, UK) containing 80% N₂, 10% CO₂ and 10% H₂ for 18-24 h, while aerobic bacteria were incubated at 35°C for 18-24 h in air.

2.2.2 Standardization of inocula

Inocula were prepared according to CLSI guidelines^{164,165}. Briefly, bacterial suspensions were made by suspending the growth from a 24-48 h cultures in 0.85% saline. Inocula were standardized using a nephelometer (Hach Company, Loveland, CO, USA). Suspensions for *C. difficile* isolates were adjusted to 2 McFarland (a percentage transmission value (T) of 50-60% on the nephelometer), which corresponded to approximately $1-4 \times 10^7$ colony forming unit/ml (CFU/ml). Suspensions for the remaining

Table 2.4 Other consumables.

Consumable	Supplier
Glasstic slide with grids	Kova International, Garden Grove, CA, USA
Microplate (96-well, polystyrene, white, opaque)	Nalge Nunc International, Roskilde, Denmark
Microplate (96-well, UV transparent)	Molecular Devices, Sunnyvale, CA, USA
Microscope Slides	Knittel Glasser, Braunschweig, Germany
Microtiter trays (24-well, polystyrene, clear)	Sigma Aldrich, Castle Hill, NSW, Australia
Microtiter trays (96-well, polystyrene, clear)	Thermo Fisher Scientific, Scoresby, VIC, Australia
Plastic petri dishes, 90 mm	Biolab, East Tamaki, New Zealand
Sterile syringe filter, Acrodisc 0.22 μm	Acrodisc [®] Pall corporation, MI, USA
Syringe 5cc/ml	Becton Dickson, Franklin Lakes, NJ, USA

bacteria were adjusted to 0.5 McFarland (%T value of 85), corresponding to approximately 1×10^8 CFU/ml.

2.2.3 Viable counts

Viable counts were performed using the Miles-Misra drop technique and spread plate methods. Each sample was diluted in a series of 10-fold dilutions using the diluent stated for each particular assay. For anaerobic bacteria, the agar plates were pre-reduced for 3-4 h in the anaerobic chamber prior to inoculation, while for aerobic bacteria the agar plates were pre-dried for ~ 30 min prior to use.

Miles-Misra technique

Miles-Misra counts were performed by spot-inoculating two replicate 20 μ l drops onto the agar surface ¹⁶⁶. After the spots had dried, plates were incubated either anaerobically at 35°C for 24-48 h or aerobically at 35°C overnight. The limit of detection (LOD) is stated in the relevant sections for each assay.

Spread plates

Spread plates were performed by spreading 100 μ l of the appropriate dilution over the surface of an agar plate using a sterile hockey stick. Duplicate plates were used for all occasions and only plates with 30-300 colonies were counted.

2.2.4 Preparation of natural products and antimicrobial agents

To prepare solutions or extracts for testing raw products such as fresh garlic, ginger and onion were washed with SDW, peeled and crushed using a mortar and pestle or a grinder. To obtain the extract, the crushed products were then filtered through cheesecloth and the filtrate retained. The honey was prepared (w/v) in SDW and solutions were held at 37°C

for 15 min to aid mixing. The dried powdered spices were suspended in either SDW or 20% DMSO and were shaken gently at room temperature overnight. Afterward, the spice suspensions were centrifuged and the supernatant retained for testing. One portion of each sample was passed through a 0.2 μm pore filter to remove contamination and the remainder was tested without filtration. The processed products and the pure compounds (Table 2.2) were either used neat or suspended in 20% DMSO. Since the processed products were assumed to contain no microbial contamination, they were not filter-sterilized.

2.3 ANTIMICROBIAL SUSCEPTIBILITY ASSAYS

2.3.1 Agar well diffusion assay

To prepare the inocula, anaerobic bacteria were cultured on pre-reduced SBA and plates were incubated in the anaerobic chamber at 35°C for 36-48 h, while aerobic bacteria were cultured on BA for 18-24 h at 35°C. Growth was suspended in pre-reduced 0.85% saline for anaerobic bacteria and non-reduced 0.85% saline for aerobic bacteria, and was standardized as described in Section 2.2.2.

Pre-reduced SBA or MHA was lawn inoculated with a cotton-tipped swab. Wells of 8 mm were cut into each inoculated agar plate and a 100 μl aliquot of each unfiltered solution was pipetted into each well. The effect of filtration on antimicrobial activity was also assessed using raw products. For this, one portion of each product was passed through a 0.2 μm pore filter before being added into the well, while the other portion was added to the well without filtration. A maximum of four products were tested per agar plate. Tests with anaerobic bacteria were incubated in the anaerobic chamber at 35°C for 48 h and tests with aerobic bacteria were incubated at 35°C for 18-24 h. After incubation, zones of growth inhibition were measured to the nearest millimeter. SDW and the highest concentration of DMSO used in the experiments were included as negative controls.

Vancomycin (30µg/disc) and trimethoprim (5µg/disc) were used as positive controls for *C. difficile* and aerobic bacteria, respectively. The assay was repeated at least three times, and the mean and standard deviation (SD) calculated for zones of growth inhibition.

2.3.2 Broth microdilution assays

Minimum inhibitory concentrations (MICs) for natural products and antimicrobial agents were determined by the broth microdilution method according to the CLSI guidelines with slight modifications^{164,165}.

2.3.2.1 MIC determination for log-phase *C. difficile* cultures

Each *C. difficile* strain was cultured on BA for 48 h and the growth suspended in pre-reduced BHIB, which was incubated in the anaerobic chamber at 35°C until the cells reached mid-exponential phase (approximately 12 h, OD₆₀₀ ≈ 0.5).

TY broth was used for the broth microdilution assay to mimic the conditions used in the sporulation assay. The MICs of products against log-phase *C. difficile* cultures were used mainly in the sporulation assay. The mid-log phase *C. difficile* cultures were inoculated into TY broth containing doubling dilutions of natural products and antimicrobial agents in a 96-well microtiter tray to give a final inoculum concentration of approximately 1×10^6 CFU/ml. The total volume for each well was 200 µl with a 1:1 ratio of cells and test compounds. Microtiter trays were incubated anaerobically at 35°C for 48 h and MICs were determined visually using a reading mirror at 24 h and 48 h as the lowest concentration of product preventing growth and resulting in no turbidity. To control for the effect of DMSO on the growth of bacteria, doubling dilutions of 20% DMSO were prepared and tested in parallel. This served as a solvent control. Fidaxomicin was also included as an antimicrobial control.

2.3.2.2 MIC and minimum bactericidal concentration (MBC) determination for stationary-phase *C. difficile*

The inocula were prepared as described in Section 2.2.2. For each strain, a suspension in pre-reduced SBB was prepared to contain 1×10^6 CFU/ml and used to inoculate serial dilutions of natural products and antimicrobial agents prepared in 96-well microtiter trays. Each well contained a total volume of 200 μ l with a 1:1 proportion of inocula and treatments. After incubation for 48 h, the MIC values were determined as described in Section 2.3.2.1. MBCs were determined by spot-inoculating 10 μ l volumes from non-turbid wells onto pre-reduced BA. Agar plates were incubated for 24-48 h anaerobically and MBCs were determined as the lowest concentration preventing growth. The 20% DMSO solution was serially diluted two-fold and tested for all strains in each experiment. This assay was repeated at least three times and modal values were determined for MICs and MBCs.

2.3.3 Determination of antibacterial synergy using a checkerboard method

Checkerboard testing was performed to determine the effect of combining natural therapeutic agents with the antimicrobials vancomycin or metronidazole against *C. difficile* ATCC 700057 and *C. difficile* NCTC 13366. A 96-well microtiter tray was used with the first agent serially diluted across each row and then serial dilutions of the second agent added to each column. Therefore, each row and column contained a fixed concentration of one agent and decreasing concentration of the other agent. Each tray contained one row and one column with the serial dilution of each agent alone. The remainder of the assay, including inoculation, incubation and endpoint determination was performed as described in Section 2.3.2.2. The fractional inhibitory concentration (FIC) was calculated for each treatment in each combination. The equation used for calculating the FIC index was as follows: $FIC_{\text{antimicrobial}} = MIC_{\text{antimicrobial combination}} / MIC_{\text{antimicrobial}}$

alone, $FIC_{\text{natural product}} = MIC_{\text{natural product combination}} / MIC_{\text{natural product alone}}$ and FIC index = $FIC_{\text{index}} = FIC_{\text{antimicrobial}} + FIC_{\text{natural product}}$. The FIC index was interpreted as synergistic ($FIC \leq 0.5$), no interaction ($FIC > 0.5 - 4.0$) or antagonistic ($FIC > 4.0$)¹⁶⁷. Results were also expressed graphically using isobolograms. The assay was repeated on three different occasions.

2.4 SPORE-RELATED ASSAYS

2.4.1 Sporulation in liquid broth cultures

The effect of natural products on sporulation in liquid culture was carried out as described previously¹⁶⁸ with slight modifications. For each of the four *C. difficile* strains, log-phase cultures were inoculated into TY broth containing $0.5 \times MIC$ of each treatment to obtain a final concentration of 1×10^6 CFU/ml. The assay was performed in duplicate 96-well microtiter trays with a total volume of 200 μ l per well. Untreated *C. difficile* controls were also included. The microtiter trays were incubated anaerobically at 35°C for 96 h. One tray was assessed at 48h whilst the duplicate was assessed at 96h. After 48 h and 96 h of incubation, 50 μ l aliquots from each well were serially diluted and plated on BHIA-tag and the total CFUs was counted after 24-48 h. Moreover, to kill any vegetative cells, 50 μ l aliquots from each sample were added to 50 μ l of 100% ethanol (50% final concentration), treated for 1 h and then plated on BHI-tag using the Miles-Misra technique (described in Section 2.2.3) to quantify viable spores after 24-48 h. The limit of detection was 500 CFU/ml. The mean CFU/ml was calculated from three experiments and the percentage of spores was determined by dividing the number of spores by the total viable count. Also, another 50 μ l volume from each sample was sub-cultured on BHIA without taurocholate and glycine, as spores germinate poorly in the absence of germinants. This was used to monitor the viability of vegetative cells only.

2.4.2 Microscopic evaluation of sporulation

The assay was performed as described previously¹⁶⁸. Aliquots of 15 µl of each bacterial suspension grown in TY broth from the sporulation assay in liquid broth cultures (Section 2.4.1) were deposited onto microscope slides, air-dried and heat fixed at 80°C. The slides containing the fixed samples were stained with 0.5% aqueous safranin and malachite green, and observed via light microscopy. For each slide, five different fields of view, each containing at least 30 cells (minimum of 150 cells per slide) was used to count the numbers of vegetative cells and spores. The percentage of spores was calculated for each treatment and the mean and SD for three independent replicates were determined. Initially, all natural products were tested against *C. difficile* NCTC 13366 and both the conventional spore recovery assay and microscopic methods were used to count the spores and vegetative cells. Products that showed reduced sporulation against *C. difficile* NCTC 13366 were tested further against the remaining three *C. difficile* strains. SDW and the highest concentration of DMSO used in the experiment were included as untreated, negative controls (with no effect on sporulation), while fidaxomicin was used as a positive control (inhibiting sporulation).

2.4.3 Preparation of spore suspensions

Spore suspensions were prepared according to the method described by Moore *et al.*¹⁶⁹ with slight modifications. For each of the four *C. difficile* strains, a 48 h culture on pre-reduced BA was prepared, from which colonies were sub-cultured onto pre-reduced chocolate agar and incubated anaerobically at 35°C for 10 days. Cultures were then left at room temperature for 24 h to kill any vegetative cells. Growth from each plate was then suspended in cold SDW. The bacterial suspensions were incubated at 70°C in a water bath for 20 min to ensure that no viable vegetative cells remained. Subsequently, the suspensions were centrifuged at $4000 \times g$ for 10 min, the pellet was washed at least 10

times in SDW and then re-suspended in SDW. To ensure a spore purity of >95%, the spore suspensions were examined using phase-contrast microscopy. The spore count was also determined by serially diluting and culturing the spore suspensions on Chrom-ID *C. difficile* agar. Spore suspensions were stored at -20°C until use.

2.4.4 Spore germination and outgrowth assay

The effects of natural products on spore germination were examined using a previously described assay ¹⁷⁰. Briefly, spores (Section 2.4.3) of all four *C. difficile* strains were suspended to a final concentration of 1×10^6 CFU/ml in pre-reduced BHIB supplemented with 5 g/L yeast extract, 0.1% L-cysteine and 0.1% taurocholate containing natural products at supra-MIC concentrations (Table 4.1). Spore germination and outgrowth was measured by determining the absorbance at 600nm (A_{600}) at time zero, every 5 min for the first 30 min, every 10 min for the next 30 min, and then at 2, 3, 4, 5, 6 and 24 h, using a Bio-Rad xMark spectrophotometer (Bio-Rad, Hercules, CA, USA). The assay was performed in 96-well trays incubated anaerobically at 35°C between measurements. The assay was repeated at two-fold and four-fold lower concentrations for treatments that showed an effect on either the germination or outgrowth stage. To normalize germination and outgrowth, the optical density (OD) measured at each time point was divided by the OD at time zero (t_0).

Both SDW and the highest concentration of DMSO used in the experiment were included as untreated controls (affecting neither *C. difficile* germination nor outgrowth). Fidaxomicin and vancomycin were included as positive controls (inhibiting *C. difficile* outgrowth). Three biological repeats of the assay were performed, and the mean and SD determined.

2.4.5 Sporicidal activity assay

Sporicidal activity was evaluated as previously described¹⁷¹. Briefly, purified spore suspensions (see Section 2.4.3) were diluted in 0.85% saline to result in a final concentration of 1×10^6 CFU/ml in a total volume of 5 ml after the addition of treatments. Suspensions were incubated anaerobically at 35°C for 24 h, 48 h and 1 week and 1 ml aliquots were removed at each time point. Spores were recovered by centrifuging suspensions for 10 min at $4000 \times g$ and washing twice in 0.85% saline to minimize carryover of any residual products. The washed spore pellets were suspended in 1 ml of 0.85% saline, serially diluted and plated on BHI-tag agar using the spread plate method described in Section 2.2.3. The number of CFUs was determined after 24-48 h anaerobic incubation at 35°C. Negative controls consisted of SDW and the highest concentration of DMSO. The LOD was 5×10^2 CFU/ml for this assay. The assay was repeated three times and the mean and SD values determined.

2.5 TOXIN ACTIVITY ASSAYS

2.5.1 Toxin protection assay

The toxin protection assay was performed to investigate whether products could protect cells from the cytotoxic effect of *C. difficile* toxins.

2.5.1.1 Tissue culture

The assay was performed as described previously¹⁷² with slight modifications. Vero and HT-29 cells were passaged in a 96-well microtiter tray and incubated for 24 h at 37°C in a 5% CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA) prior to use. To obtain confluent monolayers, Vero and HT-29 cells were added into 96-well microtiter trays at 6×10^4 cells/ml and 5×10^5 cells/ml, respectively and incubated for 24 h.

2.5.1.2 Culture filtrate preparation

C. difficile strains were cultured on pre-reduced BA for 48 h, after which growth was suspended in pre-reduced 0.85% saline to prepare bacterial suspensions equivalent in turbidity to a 2 McFarland turbidity standard. An aliquot of 100 μ l from each suspension was inoculated into 10 ml of pre-reduced BHIB and incubated anaerobically at 35°C for 5 h. Following incubation, the broth cultures were aseptically sealed and incubated for a further 5 days at 37°C in a Thermocube incubator (Bioline, Narellan, NSW, Australia) with shaking at 120 revolutions per min (rpm). Cultures were then centrifuged at 4,000 $\times$ g for 5 min and the supernatant containing toxin separated from cells and debris by filtering through a 0.22 μ m-pore size Millipore filter.

2.5.1.3 Toxin titration

Serial two-fold dilutions of culture filtrates in DMEM-MM were prepared and 100 μ l of each dilution was added to each well containing Vero or HT-29 cells. An inverted microscope (Olympus Corporation, Tokyo, Japan) was used to determine the 90% cytotoxicity titer at 400 $\times$ magnification. All titrations were performed three times and a modal value was determined.

2.5.1.4 Preparation of treatments

Natural products and fidaxomicin were prepared as described in Section 2.2.4. Serial two-fold dilutions of natural products, fidaxomicin and DMSO were prepared in DMEM-MM and 100 μ l of each dilution added to Vero cell monolayers. The highest concentrations showing no visible effect on cells after 24 h incubation, determined by microscopy, were used in the subsequent toxin protection assay.

2.5.1.5 Incubation of treatments and culture filtrates

The highest concentrations of products with no visible effect on cell monolayers, in addition to 2-fold and 4-fold lower concentrations, were used in this assay. Those products that showed protection against cytotoxicity on Vero cells were also tested on HT-29 cells. To account for the dilution factor, the supernatants from the three toxigenic *C. difficile* strains were prepared at a concentration 4-fold higher than the minimum dilution required to achieve 90% cell rounding. The culture filtrates and products were both diluted in DMEM-MM. To evaluate whether natural products reduced the activity of free toxin, diluted antimicrobial agents and supernatants were mixed at a ratio of 1:1 and incubated at 37°C for 2 h. Aliquots of 100 µl of 1:1 antimicrobial agents: culture filtrates were added to 96-well microtiter plates containing Vero cell monolayers and incubated in the 5% CO₂ incubator at 37°C for 24 h. An inverted microscope was used to evaluate protection from CPE and results were verified with a neutral red uptake assay (see Section 2.5.1.6).

Culture filtrates from the non-toxigenic strain *C. difficile* ATCC 700057 and the three other toxigenic strains without the addition of treatments were used as controls (the three toxigenic strains were expected to show CPE but not the non-toxigenic strain). The assay was repeated on three different occasions. If protection or a reduction in cytotoxicity was shown by any products, these were further investigated to assess whether they blocked the binding sites or inactivated the toxin. To investigate the ability of products to block cell binding sites, the cell monolayers were pre-incubated for 2 h with treatments at 2-fold higher than the desired concentrations at 37°C with 5% CO₂, followed by the addition of an equivalent volume of *C. difficile* culture filtrate. Furthermore, to evaluate whether protection occurred immediately or pre-incubation was required, diluted treatments and culture filtrates were mixed in equal volumes and 100 µl of the mixture was immediately transferred to the wells containing cell monolayers. Cytotoxicity was evaluated using

microscopy and the results were further confirmed by a neutral red assay as described in the next section.

2.5.1.6 Neutral red uptake assay

To confirm the visual perception of protection from CPE and to determine the cell viability, a neutral red uptake assay was performed ¹⁷³. Following the visual evaluation of cytotoxicity results after 24 h of incubation, the contents of each well were removed and a 100 μ l aliquot of 40 μ g/ml neutral red solution was added. Plates were incubated for 3 h at 37°C with 5% CO₂, and wells were then washed with 150 μ l of phosphate buffered saline (PBS pH 7.3). Subsequently, 150 μ l of neutral red destain solution was added to each well and the plates placed on a shaker at 120 rpm for 10 min to elute the stain. The OD of each well was determined at 540nm (xMark™ microplate spectrophotometer). Wells without cells served as blanks. The untreated control cells were assumed to be 100% viable, and the percentage of viable cells in wells containing treated and untreated culture filtrates was calculated relative to the untreated control cells. The assays were repeated on three different occasions and the mean and SD determined.

2.5.2 Indirect toxin-mediated cytotoxicity assay

The indirect effect of natural products on the activity of *C. difficile* toxins was determined as described previously ¹⁷⁴. Initially, the effect of all products against toxin-mediated cytotoxicity was assessed using Vero cells and those products showing any effect against toxin activity on Vero cells were further tested with HT-29 cells. Briefly, a 5 ml suspension at a concentration of 1×10^6 CFU/ml was prepared for each strain in pre-reduced BHIB containing $0.5 \times \text{MIC}$ and $0.25 \times \text{MIC}$ of treatments. After incubating the cultures anaerobically at 35°C for 48 h, samples were centrifuged at $4,000 \times g$ for 10 min and the culture was filtered through a 0.22 μ m pore-size membrane filter. Prior to being

added to the cell monolayer plates, the culture filtrates were diluted 1:100 to reduce the gross effect of natural products on cell monolayers. The diluted culture filtrates were further serially diluted 1:2 in 96-well microtiter plates containing cell monolayers using DMEM-MM. The plates were examined under an inverted microscope following 48 h incubation in CO₂ at 37°C. The reciprocal of the highest dilution causing 90% cell rounding was expressed as the cytotoxicity titer. All natural products were tested against *C. difficile* NCTC 13366 and *C. difficile* R11446 using Vero cells and those products showing a reduction in cytotoxicity titer were further tested against *C. difficile* ATCC 43598. Only relatively minor CPE was observed with *C. difficile* ATCC 43598 on HT-29 cells, therefore, only *C. difficile* NCTC 13366 and *C. difficile* R11446 were tested on those cells. The culture filtrate from *C. difficile* ATCC 700057 was used as a negative control (showing no CPE) in this assay and fidaxomicin was used as a positive control (reducing CPE). The assay was repeated on three different occasions in duplicates and a modal cytotoxicity titer was determined.

2.5.3 Effect of treatments on toxin production

The amount of toxin in cultures grown in the presence of $0.5 \times \text{MIC}$ and $0.25 \times \text{MIC}$ of treatments relative to the amount of toxin in control cultures (grown without antimicrobials) was determined using the *C. difficile* TOX A/B IITM ELISA kit (TECHLAB[®] Blacksburg, VA, USA). Only products showing activity in either toxin activity assays were included in this assay. Also, fidaxomicin was included as an antimicrobial control. Culture filtrates from the three toxigenic *C. difficile* strains were prepared as described in Section 2.5.1.2. Subsequently, the culture filtrates were diluted and tested according to the manufacturer's instructions. The spectrophotometer was blanked against air at a wavelength of 620nm and the OD was measured at 450nm. Relative toxin production was expressed as a percentage of OD₄₅₀ for treated culture

filtrates over OD₄₅₀ of untreated culture filtrates. The assays were repeated three times and the mean and SD determined.

2.6 INVESTIGATING THE MECHANISMS OF ACTION OF NATURAL PRODUCTS

2.6.1 Time-kill assays

The assay was carried out as described by Wu *et al.* with minor modifications¹⁷⁵. Time-kill kinetics for bactericidal products were determined against both log-phase and stationary-phase cultures of *C. difficile* ATCC 700057 and *C. difficile* NCTC 13366.

Log-phase and stationary-phase inocula were prepared as described in Sections 2.3.2.1 and 2.3.2.2, respectively. The only exception was that growth was adjusted to be equivalent to 1×10^8 CFU/ml. The cultures in pre-reduced SBB were exposed to 1, 2, 4 and $8 \times$ MIC of products with a final inoculum concentration of $\approx 1 \times 10^7$ CFU/ml. The total final volume for each suspension, including the treatments, was 5 ml. Samples were taken at 0, 1, 2, 4, 6 and 24 h, serially diluted ten-fold in 0.85% saline and 20 μ l volumes of each dilution was spot-inoculated onto pre-reduced SBA. After 24-48 incubation anaerobically at 35°C, colonies were counted and the number of surviving organisms was determined using the Miles-Misra method described on Section 2.2.3. The LOD was 5×10^2 CFU/ml. Both SDW and the highest concentration of DMSO used in the experiment were included as untreated controls. The assay was performed on three different occasions and the mean and SD determined.

2.6.2 ATP-leakage assay

The ATP-leakage assay was performed according to both Wu *et al.*¹⁷⁵ and the instructions included in the BacTiter-Glo™ kit (Promega, Madison, WI, USA). The log-phase culture (12 h, OD₆₀₀ $\approx$ 0.5) of *C. difficile* NCTC 13366 was prepared as described in Section

2.3.2.1. The cultures in pre-reduced SBB were exposed to 1, 2, 4 and $8 \times \text{MIC}$ of products with a final concentration of $\approx 1 \times 10^7$ CFU/ml. The final volume for each bacterial suspension was 5 ml after the addition of treatments, which were incubated in the anaerobic chamber at 35°C.

Samples of 1 ml were taken at 1, 2 and 6 h for the determination of the intracellular and extracellular levels of ATP using the BacTiter-Glo™ kit (Promega, Madison, WI, USA). The samples were centrifuged at $10,000 \times g$ for 10 min; the intracellular ATP was determined from the cell pellets resuspended in pre-reduced BHIB, while the extracellular ATP was determined from the supernatant. The luminescence was measured using the FluoStar Optima Plate Reader (BMG Labtech, Pretoria, South Africa) and the ATP level over time was expressed as a percentage of the untreated control (SDW). The assay was performed three times and the mean and SD determined.

2.6.3 Bacteriolysis assay

The bacteriolysis assay was carried out as described previously¹⁷⁶ with slight modifications. *C. difficile* ATCC 700057 and NCTC 13366 were cultured on pre-reduced BA for 24-36 h to reach stationary phase. Then, growth was suspended in pre-reduced PBS (pH 7.3). Each cell suspension contained a final concentration of $\approx 1 \times 10^8$ CFU/ml, containing the highest achievable concentration of products (range $2 \times \text{MIC}$ - $8 \times \text{MIC}$) with a final volume of 5 ml. The suspensions were mixed immediately using a vortex mixer, diluted 1 in 10 and the OD₆₂₀ was measured (time zero). The treatments and controls were incubated in the anaerobic chamber at 35°C. Additional samples were taken at 1, 2 and 6 h. Samples were diluted 1 in 10 and the absorbance was read in triplicate against the appropriate blanks (corresponding dilutions of test agents prepared as above, though without inoculum). Vancomycin and Triton X-100 were used as negative and positive controls, respectively. SDW and the highest concentration of DMSO used in the

experiment were included as untreated controls. The results were expressed as a percentage of OD₆₂₀ at each time point over the OD₆₂₀ at time 0 h. The assay was repeated on three different occasions and the mean and SD determined.

2.6.4 Assays to determine alterations in cell permeability

The two *C. difficile* strains ATCC 700057 and NCTC 13366 were used for the cell permeability assays. The bacterial suspensions were prepared as described in Section 2.2.2 and exposed to treatments at several concentrations (1× MIC, 2 × MIC, 4 × MIC and 8 × MIC). Each bacterial suspension had a final volume of 5 ml after the addition of treatments. For all assays, vancomycin (8 µg/ml), SDW and the highest concentration of DMSO were used as negative controls, while nisin (4 µg/ml) was included as a positive control. Each assay was repeated on three different occasions and the mean and SD determined.

2.6.4.1 Leakage of 260nm-absorbing material

The assay was performed as previously described by Carson *et al.*¹⁷⁶. The bacterial suspensions in PBS (pH 7.3) were exposed to different concentrations of treatments and were incubated anaerobically at 35°C. At time 0, 1, 2 and 6 h post-incubation, 1 ml aliquots of each sample were removed, diluted 1 in 10 in PBS (pH 7.3) and filtered through a 0.22-µm pore size filter. For each filtrate, the absorbance was measured in triplicate (three separate aliquots read once) at 260nm. Corresponding filtrates of the appropriate dilution of each treatment were prepared and used as blanks. Aliquots of 200 µl were added to wells of a UV-transparent 96-well tray (Molecular Devices, Sunnyvale, CA, USA), which was used for both this and the bacteriolysis assays. The OD was measured using an xMarkTM microplate spectrophotometer.

2.6.4.2 Protein leakage assay

Extracellular protein was quantified using the Bradford protein assay ¹⁷⁷. The bacterial suspensions were prepared in PBS (pH 7.3). At time 0, 1, 2 and 6 h post-incubation anaerobically with treatments at 35°C, 1ml aliquots were removed from each treatment. The samples were centrifuged at 6000 rpm for 10 min and the supernatants were collected. For each sample, 100 µl of supernatant was mixed with 100 µl of Bradford working solution in a 96-well microtiter tray. The OD₅₉₅ was measured in triplicate after a 10 min incubation in the dark. Supernatants of the appropriate dilution of each treatment alone were prepared and used as blanks. A standard curve was constructed using a series of concentrations of BSA as a standard protein with a known concentration (Figure 2.1). The concentration of extracellular protein was determined via the linear regression line of the standard curve using the absorbance values.

2.6.4.3 Propidium iodide uptake

The protocol described by Wu *et al.* ¹⁷⁵ was used for this experiment with minor modifications. Stationary-phase cells in BHIB were exposed to different concentrations of treatments and incubated anaerobically at 35°C. At time 1, 2 and 6 h, 1 ml aliquots were removed from each treatment, centrifuged at 6000 rpm for 10 min and cell pellets were re-suspended in 1 ml propidium iodide working solution (5 µM). Following a 10 min incubation anaerobically at 35°C, samples were centrifuged and washed twice with PBS to remove the excess dye. Propidium iodide fluorescence in cells was measured using a FluoStar Optima Plate Reader (BMG Labtech, Pretoria, South Africa) at an excitation wavelength of 544nm and an emission wavelength of 620nm.

Figure 2.1 Standard curve of BSA (mg/ml) measured at OD₅₉₅. The protein leakage from bacterial cells was determined by the linear formula: $y = 0.0118x + 0.047$.

2.6.5 Protein synthesis inhibition assay

An *in-vitro* transcription/translation experiment was carried out using the *E. coli* S30 extract system for circular DNA and the Steady-Glo[®] Luciferase assay system (Promega), in accordance with the manufacturer's instruction. The assay was performed on fresh garlic extract, *Leptospermum* honey (MGO 514+) and allicin, which demonstrated bacteriostatic activity against tested *C. difficile* strains. Streptomycin sulfate and tetracycline were included as positive controls known to inhibit protein synthesis. Briefly, a master mix was prepared by combining the reaction components included in the kit. Each reaction contained 49 µl of the master mix and 1 µl of the treatment (Table 6.6) and was incubated at 37°C for 1 h. The reaction was then stopped by placing the tubes on the ice for 5 min. To detect the luciferase protein, 95 µl of SDW was added to 5 µl of each translation reaction in a white opaque 96-well microplate, followed by addition of 100 µl of Steady-Glo[®] reagent. Luminescence was measured in terms of the relative light unit (RLU) using the FluoStar Optima Plate Reader (BMG Labtech, Pretoria, South Africa). The net luminescence was calculated by subtracting the background luminescence (containing only SDW) from the experimental luminescence. The relative inhibition of protein synthesis was expressed as a percentage of net luminescence in the presence of treatment over the net luminescence in absence of treatments. The assay was repeated three times and the mean and SD determined.

2.6.6 Indirect assessment of mechanism of antimicrobial resistance

To determine whether antimicrobial resistant (AMR) bacteria that have reduced susceptibility (overlapping mechanisms of action) to products showing either bactericidal or bacteriostatic activity, a broth microdilution assay was carried out comparing the MICs of products against a panel of *C. difficile* strains previously characterized for AMR phenotype and associated genotype¹⁷⁸. The panel comprised strains determined to be (i)

fluoroquinolone resistant (FQR), mediated by DNA gyrase mutations; (ii) tetracycline resistant (tetR), mediated by conjugative transposons Tn5397 and Tn6190 carrying *tetM*; and (iii) resistant to erythromycin and clindamycin (macrolide-lincosamide-streptogramin B (MLS_B)) phenotype, mediated by conjugative transposon Tn6194 carrying *ermB*. The MIC of each product against these strains was determined on three separate occasions and a modal value determined. The metronidazole and vancomycin-resistant isolates were prepared using the serial passaging method detailed in the next section. Tables 2.5 and 2.6 summarise the antimicrobial phenotypes and genotypes for the selected *C. difficile* isolates as well as their antibiograms.

2.6.6.1 Serial passage assay

The assay was performed as described previously¹⁷⁹ with slight modifications. The serial passage of *C. difficile* isolates ATCC 700057 and NCTC 13366 was performed in the presence of increasing concentrations of metronidazole and vancomycin. The initial inoculum was prepared and MICs were determined as described in Sections 2.2.2 and 2.3.2.2, respectively. However, 24-well microtiter trays were used instead of 96-well trays with a total volume of 1 ml per well comprising a 1:1 proportion of cells and antimicrobial agents. Post-incubation, serial passaging was initiated by removing the contents of well with the highest concentration of antimicrobial with turbidity (~ 1 ml) and adding to 4 ml of pre-reduced 2 × strength SBB. This inoculum was used to inoculate the next MIC assay by adding 0.5 ml of inoculum suspension to 0.5 ml of each antimicrobial agent and concentrations were adjusted if required. The 24-well trays were incubated anaerobically at 35°C for 48 h, and the process was repeated 20 times. Each antimicrobial/bacterium combination was tested in duplicate. Finally, organisms were recovered from the 20th passage by streaking for single colonies and the cells were stored

Table 2.5 Antimicrobial phenotypes and genotypes for selected *C. difficile* isolates.

Isolate ID	Host	RT	ST (clade)	Phenotype	Genotype	MIC (mg/L)				Genbank accession	Ref
						CLI	ERY	MXF	TET		
ESP123	Porcine	014	13 (1)	MLS _B /tetR	Tn6194/Tn5397	>32	>256	1	32	ERS1078745 ^a	178
SQ383	Human	014	13 (1)			0.12	1	2	0.06	ERS1078755 ^a	178
SQ357	Human	014	2 (1)	MLS _B	Tn6194	8	>256	2	0.06	ERS1078753 ^a	178
SQ463	Human	014	2 (1)			2	1	2	0.06	ERS1078757 ^a	178
SAP019	Porcine	014	2 (1)	MLS _B /tetR	Tn6194/Tn5397	16	>256	1	32	ERS1078759 ^a	178
SQ317	Human	014	2 (1)			0.12	0.06	0.5	0.06	ERS1078752 ^a	178
ES0474	Human	126	11 (5)	FQR/tetR	<i>gyrAB</i> mutations/Tn6190	1	1	16	16	ERS2546360 ^b	This study
ES1046	Human	126	11 (5)			0.12	0.5	1	0.06	ERS2546361 ^b	This study

RT, ribotype; ST, multi-locus sequence type; CLI, clindamycin; ERY, erythromycin; MXF, moxifloxacin; TET, tetracycline; MLS_B, macrolide-lincosamide-streptogramin-B; tetR, tetracycline resistant; FQR, fluoroquinolone resistant. Blue and red colored values indicate susceptible and resistant MIC values, respectively. ^aEuropean Nucleotide Archive (ENA) study PRJEB12970. ^bENA study PRJEB27277.

Table 2.6 Antibigrams for selected *C. difficile* isolates.

Isolate ID	Host	RT	Phenotype	VAN	MET	FDX	RFX	CLI	ERY	AUG	CRO	MEM	MXF	TET	TZP	TMP
ESP123	Porcine	014	MLS _B /tetR	1	0.25	0.06	0.004	>32	>256	0.25	16	2	1	32	8	32
SQ383	Human	014		1	0.5	0.008	0.004	0.12	1	0.25	8	1	2	0.06	4	8
SQ357	Human	014	MLS _B	1	0.5	0.008	0.004	8	>256	0.25	16	2	2	0.06	4	32
SQ463	Human	014		1	0.5	0.015	0.004	2	1	0.25	16	1	2	0.06	8	16
SAP019	Porcine	014	MLS _B /tetR	1	0.25	0.03	0.004	16	>256	0.25	16	2	1	32	8	32
SQ317	Human	014		0.5	0.12	0.004	0.002	0.12	0.06	0.12	8	0.5	0.5	0.06	0.5	16
ES0474	Human	126	FQR/tetR	1	0.5	0.03	0.008	1	1	0.25	16	2	16	16	4	64
ES1046	Human	126		1	0.5	0.004	0.004	0.12	0.5	0.25	16	1	1	0.06	8	8

RT, ribotype; VAN, vancomycin; MET, metronidazole; FDX, fidaxomicin; RFX, rifaximin; CLI, clindamycin; ERY, erythromycin; AUG, augmentin; CRO, ceftriaxone; MEM, meropenem; MXF, moxifloxacin; TET, tetracycline; TZP, piperacillin-tazobactam; TMP, trimethoprim; MLS_B, macrolide-lincosamide-streptogramin-B; tetR, tetracycline resistant; FQR, fluoroquinolone resistant.

immediately at -80°C.

2.7 THE EFFICACY OF PRODUCTS WITH POTENT *IN-VITRO* ACTIVITY IN A MOUSE MODEL OF CDI

2.7.1 Ethics approval

The mouse trial presented in Chapter 7 was conducted by Professor Dena Lyras' research group and was approved by the Animal Ethics Committee (AEC) at Monash University in Melbourne (approval no. MARP/2014/142). Reciprocal approval was obtained from the AEC at UWA.

2.7.2 Study Design

Two pure compounds, zingerone and *trans*-cinnamaldehyde were chosen on the basis of their potent *in-vitro* activity for testing in a mouse model of CDI^{180,181}. Mice were pre-treated with an antimicrobial cocktail for 10 days to disrupt the resident microbiota and induce susceptibility to infection with *C. difficile*. On the day of infection, antimicrobial pre-treatment was ceased. Groups of five mice were infected with 10⁵ *C. difficile* M7404 spores. Previous *in-vivo* studies investigating the effect of either zingerone or *trans*-cinnamaldehyde in animal species such as mice and rats were reviewed to determine an appropriate dose for the mice (Table 2.7). Mice were subsequently treated with either 10% ethanol control, zingerone (500 mg/kg/day) or *trans*-cinnamaldehyde (1250 mg/kg/day) by oral gavage at 6 h post infection, then compounds were given *ad libitum* in their drinking water at the same concentrations. The mice were monitored daily for signs of infection and faecal samples were analyzed for the presence of *C. difficile* spores as follows. Faecal pellets were resuspended in PBS (100 mg/ml), heat shocked for 30 min at 65°C and plated on supplemented heart infusion (HIS) agar containing 0.1% (w/v) sodium taurocholate 250 µg/ml D-cycloserine (Sigma Aldrich), 8 µg/ml cefoxitin

Table 2.7 Safety data for *trans*-cinnamaldehyde and zingerone for animal species.

Products	Animal	Toxicity	Administration route	Ref
<i>trans</i> -cinnamaldehyde	mouse	• 50 mg/kg was used as a treatment option in mice and no toxicity was observed.	Intraperitoneally	182
		• Ten mice were fed 8200 ppm micro-encapsulated <i>trans</i> -cinnamaldehyde and all survived a 3-months trial.	Microcapsules were combined with feed	183
		• 0.4% <i>trans</i> -cinnamaldehyde was orally administered in a mouse model for 10 days and no toxicity was observed.	In-feed supplementation	184
		• In an oral acute toxicity testing in mice, <i>trans</i> -cinnamaldehyde at 1250, 625 and 312 mg/kg body weight was administered to both male and female mice and no death or significant toxicity during the 14 day trial was observed.	Oral gavage	185
	rat	• Cinnamaldehyde has low acute oral toxicity based on animal studies. The median lethal dose (LD ₅₀) in rats was >2000 mg/kg by weight.	Oral gavage	186
		• The no-observed-adverse-effect-level (NOAEL) for non-neoplastic effects of orally administered <i>trans</i> -cinnamaldehyde was determined to be 200 mg/kg by weight per day in rats.		
		• In a 13-week study, the potential toxicity of <i>trans</i> -cinnamaldehyde was investigated in Fischer 344/N rats. Groups of 10 male and 10 female rats were given diets containing 0%, 1.25%, 2.5%, 5.0% or 10% microencapsulated <i>trans</i> -cinnamaldehyde, corresponding to 0, 620, 1250, 2500 or 5000 mg/kg by weight per day. There were no early deaths and no treatment-related clinical toxicity. From this study, the NOAEL of <i>trans</i> -cinnamaldehyde was determined to be 620 mg/kg by weight per day.	Microcapsules were combined with feed	187
zingerone	mouse	• Oral administration of freshly prepared zingerone at 500mg/kg by weight did not induce any animal mortality during the 14 days of the observation period.	Oral	188
		• Zingerone, 65 nmol/kg was administered intraperitoneally daily for 1 week to mice and no toxicity was observed.	Intraperitoneally	189
		• The median LD ₅₀ of zingerone in mice by oral administration was reported to be 657.55 mg/kg with 95% confidence intervals of LD ₅₀ ranging from 471.30 to 882.29 mg/kg.	Oral	190
	rat	• The NOAEL of zingerone in rats was estimated to be 128 mg/kg by weight per day.	Oral	191
		• LD ₅₀ in rats = 2580 mg/kg	Oral	192

LD₅₀: lethal dose, 50% or median lethal dose.

(Fluka[™] Honeywell International Inc., Morris Plains, NJ, USA), 10 µg/ml erythromycin (Amresco Inc.), 12 µg/ml norfloxacin (Sigma Aldrich) and 32 µg/ml moxalactam (Sigma Aldrich). After incubation in an anaerobic atmosphere for 48 h, the colonies were counted. The detection limit was 100 CFU/g of faeces.

2.8 STATISTICAL ANALYSIS

Arithmetic means, SDs and modal values were determined where appropriate. Statistical differences between three or more sets of data were analyzed using GraphPad Prism v.7 software (La Jolla, CA, USA) using one-way analysis of variance (ANOVA) and nonparametric technique, followed by Tukey's multiple comparison post-test if the *P* value was significant. Moreover, the Student's 2-tailed *t*-test assuming unequal variance was used to determine whether there was a significant difference between two sets of data. Toxin titers in the indirect toxin study were converted to log₂ values and titer reciprocals that were lower than 100 were converted to a lower titer reciprocal of 50 to enable data analysis. *P* values of < 0.05 were considered significant.

Chapter 3

Antimicrobial activity of natural products against *C. difficile in-vitro*

The following publication has arisen from this chapter:

Roshan N, Riley TV, Hammer KA. Antimicrobial activity of natural products against *Clostridium difficile in-vitro*. *Journal of Applied Microbiology* 2017; **123**: 92-103.

3.1 INTRODUCTION

Historically, natural products have been of major importance in identifying and developing antimicrobial agents ¹⁹³. Interest in this field has waned over time, however, the growing development of bacterial resistance to major classes of known antimicrobials has encouraged the re-evaluation of natural products to combat this problem ¹⁹⁴. Plant extracts are often comprised of various components that may act synergistically on single or multiple bacterial targets, thus contributing to the overall activity of the extract ^{195,196}. Regarding *C. difficile* specifically, there is concern about the emergence of antimicrobial resistance which may contribute to treatment failure ^{8,197}. Issues associated with the use of conventional therapies for CDI treatment have prompted the investigation of other possible treatment strategies such as natural remedies ^{197,198}. Also, combination therapy using plant extracts in conjunction with antimicrobials can expand the antimicrobial spectrum and address the problem of bacterial resistance ¹⁹⁹.

3.1.1 Aims

The aim of this study was to investigate the antimicrobial activity of 22 natural products against *C. difficile in-vitro*. Primarily, the products were screened for antimicrobial activity using an agar well diffusion assay. The MICs and MBCs of products against four *C. difficile* isolates and five intestinal commensal flora were also determined using a broth microdilution assay. Finally, antimicrobial synergy and antagonism between natural products and antimicrobials were assessed using checkerboard method.

3.2 RESULTS

3.2.1 Agar well diffusion

The natural products at their highest possible concentrations were screened against the three *C. difficile* strains, ATCC 700057, NCTC 13366 and R11446, and three comparator

strains, *S. aureus* ATCC 29213, *E. coli* ATCC 25922 and *C. perfringens* ATCC 13124 (Table 3.1). Zones of growth inhibition were observed for 14 of the 16 tested products. Of the eight raw products, all except fresh ginger rhizome extract (100% v/v) showed activity. The most active agent in the raw product group was the fresh garlic bulb extract (100% v/v). Of the eight processed preparations, all except coconut oil capsules showed activity (Table 3.1). The most active product of the seven processed preparations was the garlic tablet and the least active product was the ginger tablet. Most of the products dissolved better in 20% DMSO than in SDW and subsequently showed larger zones in DMSO than when dissolved in SDW. For instance, both ginger rhizome powder and tablets did not show any zones of inhibition when SDW was used as a solvent; whereas larger zones of inhibition were observed for ginger products (9.3-12.0 mm) when dissolved in 20% DMSO (Table 3.1). Similarly, turmeric powder (20% w/v) and tablet showed minimal activity in SDW with zones ranging from 10.0 to 11.3 mm, whereas zones were 9.0-12.5 mm when dissolved in 20% DMSO (Table 3.1).

The raw products were not sterile and may have contained organisms that would interfere with the results of the assay. Therefore, the effect of filtration on the antimicrobial activity of raw products against *C. difficile* isolates and comparators was assessed. Filtration had no significant effect on the size of inhibition zones for any of the seven raw products ($P > 0.05$) (Table 3.2). Therefore, to avoid contamination, raw products were routinely filter sterilized for the remaining antimicrobial activity assays.

3.2.2 Broth microdilution

The MICs and MBCs of products against four stationary-phase *C. difficile* strains and three comparator organisms were determined using the broth microdilution assay. Approximately 60% of natural agents at their highest concentrations showed inhibitory

Table 3.1 Screening of natural products for antimicrobial activity by agar diffusion against three *C. difficile* and three comparator strains.

Product	Solvent	Mean zone of inhibition in mm (± SD)*					
		<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 25922	<i>C. perfringens</i> ATCC 13124	<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 700057
<u>Control</u>							
Trimethoprim 5µg/disc (positive control)	-	26.1 ± 0.8	26 ± 1.0	ND	ND	ND	ND
Vancomycin 30µg/disc (positive control)	-	ND	ND	22.5 ± 1.3	30.3 ± 0.7	28.3 ± 0.9	28.7 ± 0.9
<u>Raw product</u>							
Fresh onion bulb (100% v/v)	-	10.7 ± 0.6	-	15.3 ± 0.6	10.3 ± 0.6	-	11.0 ± 0.0
Fresh garlic bulb (100% v/v)	-	42.4 ± 0.5	32.3 ± 0.9	28.7 ± 0.6	27.0 ± 1.0	26.1 ± 1.0	27.7 ± 0.6
Fresh ginger rhizome (100% v/v)	-	-	-	-	-	-	-
<i>Leptospermum</i> honey (MGO 263+) (C) (50% w/v)	SDW	15.9 ± 0.7	10.7 ± 0.4	16.5 ± 0.5	11.4 ± 0.5	10.5 ± 0.5	15.3 ± 0.6
Garlic clove powder (20% w/v)	SDW	32.3 ± 1.1	23.4 ± 0.5**	21.5 ± 0.5**	22.0 ± 0.9**	19.1 ± 0.4***	21.0 ± 0.9*
	20% DMSO	33.1 ± 1.2	25.4 ± 0.5	26.5 ± 0.7	26.6 ± 0.6	26.8 ± 0.7	24.4 ± 0.5
Ginger rhizome powder (20% w/v)	SDW	-*	-	-*	-*	-*	-*
	20% DMSO	12.0 ± 0.9	-	10.2 ± 0.7	9.6 ± 0.5	10.0 ± 0.0	10.9 ± 0.5
Cinnamon root powder (20% w/v)	SDW	16.2 ± 0.7	-*	12.0 ± 1.0**	17.8 ± 0.7**	18.6 ± 0.7*	17.3 ± 0.6**
	20% DMSO	16.2 ± 0.7	11.1 ± 0.5	16.1 ± 0.9	20.9 ± 0.9	21.0 ± 0.7	20.4 ± 0.5
Turmeric root powder (20% w/v)	SDW	-*	-*	10.0 ± 0.9	-*	-*	-*
	20% DMSO	9.0 ± 0.0	11.0 ± 0.9	10.0 ± 0.0	9.2 ± 0.7	10.0 ± 0.0	11 ± 1.0

Table 3.1 (Cont.) Screening of natural products for antimicrobial activity by agar diffusion against three *C. difficile* and three comparator isolates.

Treatment	Solvent	Mean zone of inhibition in mm (± SD)*					
		<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 25922	<i>C. perfringens</i> ATCC 13124	<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 700057
<u>Processed product</u>							
Garlic tablet (20% w/v)	SDW	20.4 ± 0.8*	14.7 ± 0.6*	-*	14.7 ± 0.6	14.0 ± 0.9	15.4 ± 0.5
	20% DMSO	21.9 ± 0.9	17.1 ± 0.9	15.5 ± 0.5	16.1 ± 0.9	14.1 ± 0.2	15.8 ± 0.2
Ginger tablet (20% w/v)	SDW	-*	-*	-*	-	-*	-*
	20% DMSO	9.3 ± 0.6	10.0 ± 0.2	10.0 ± 0.0	-	11.0 ± 0.0	9.7 ± 0.6
Cinnamon tablet (20% w/v)	SDW	-	-	10.7 ± 0.6	10.0 ± 0.9*	-	10.2 ± 0.7
	20% DMSO	-	-	11.6 ± 0.5	11.6 ± 0.5	11.9 ± 0.9	11.0 ± 1.3
Turmeric tablet (20% w/v)	SDW	-*	-*	-*	11.0 ± 0.0*	11.3 ± 0.3*	10.9 ± 0.9
	20% DMSO	9.0 ± 0.0	11.0 ± 0.2	9.0 ± 0.0	11.7 ± 0.6	12.5 ± 0.5	11.3 ± 0.6
Artichoke capsule (20% w/v)	SDW	11.7 ± 0.4	-	12.7 ± 0.6	13.1 ± 0.2	13.4 ± 0.5	12.7 ± 0.6
	20% DMSO	12.0 ± 0.9	-	13.3 ± 0.6	13.8 ± 0.3	13.9 ± 0.2	13.7 ± 0.4
Aloe Vera gel (50% w/v)	SDW	-	9.0 ± 0.0	15.3 ± 0.6**	14.2 ± 0.3	14.1 ± 0.2	15.7 ± 0.6**
	20% DMSO	-	9.0 ± 0.0	19.2 ± 0.3	15.0 ± 0.9	14.4 ± 0.2	18.8 ± 0.4
Coconut oil (100% v/v)	-	-	-	-	-	-	-
Peppermint oil (100% v/v)	-	10.6 ± 0.5	12.4 ± 0.8	11.6 ± 0.6	11.9 ± 0.2	11.6 ± 0.5	12.3 ± 0.6

SD, standard deviation; DMSO, dimethyl sulfoxide; SDW, sterile distilled water; MGO, methylglyoxal; ND, not done.

SDW and 20% DMSO were used as negative controls. **P*-values indicating the difference in activity of each product dissolved in either solvents against different isolates: * = *P* < 0.05, ** = *P* < 0.01. If no zone of inhibition was observed, an eight mm zone size was assumed to enable calculating the *P* value.

Table 3.2 Effect of filtration on antimicrobial activity of raw natural products using water as the solvent.

Treatment	Filtration	Mean zone of inhibition in mm ($\pm$ SD)					
		<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 25922	<i>C. perfringens</i> ATCC 13124	<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 700057
Fresh onion bulb (100% v/v)	-	10.7 $\pm$ 0.6	-	15.3 $\pm$ 0.6	10.3 $\pm$ 0.6	-	11.0 $\pm$ 0.0
	+	10.3 $\pm$ 0.9	-	15.7 $\pm$ 0.6	9.0 $\pm$ 0.0	-	11.5 $\pm$ 0.5
Fresh garlic bulb (100% v/v)	-	42.4 $\pm$ 0.5	32.3 $\pm$ 0.9	28.7 $\pm$ 0.6	27.0 $\pm$ 1.0	26.1 $\pm$ 1.0	27.7 $\pm$ 0.6
	+	41.4 $\pm$ 0.5	31.4 $\pm$ 0.5	27.6 $\pm$ 0.5	26.3 $\pm$ 1.0	25.3 $\pm$ 0.4	27.7 $\pm$ 0.6
Fresh ginger rhizome (100% v/v)	-	-	-	-	-	-	-
	+	-	-	-	-	-	-
Garlic clove powder (20% w/v)	-	32.3 $\pm$ 1.1	23.4 $\pm$ 0.5	21.5 $\pm$ 0.5	22.0 $\pm$ 0.9	19.1 $\pm$ 0.4	21.0 $\pm$ 0.9
	+	31.2 $\pm$ 0.7	23.2 $\pm$ 1.0	20.4 $\pm$ 0.5	22.0 $\pm$ 0.0	18.2 $\pm$ 0.9	21.1 $\pm$ 0.2
Ginger rhizome powder (20% w/v)	-	-	-	-	-	-	-
	+	-	-	-	-	-	-
Cinnamon root powder (20% w/v)	-	16.2 $\pm$ 0.7	-	12.0 $\pm$ 1.0	17.8 $\pm$ 0.7	18.6 $\pm$ 0.7	17.3 $\pm$ 0.6
	+	16.3 $\pm$ 0.6	-	12.0 $\pm$ 1.0	18.3 $\pm$ 0.4	17.7 $\pm$ 0.4	16.8 $\pm$ 0.7
Turmeric root powder (20% w/v)	-	-	-	10.0 $\pm$ 0.9	-	-	-
	+	-	-	9.0 $\pm$ 0.0	-	-	-
20% DMSO (negative control)	-	-	-	-	-	-	-

No significant difference was observed between filtered and unfiltered products.

SD, standard deviation; DMSO, dimethyl sulfoxide.

effects against all the microorganisms (Tables 3.3). Among the raw products, fresh garlic bulb extract and garlic clove powder had the highest inhibitory effect with MICs of 0.8% v/v for fresh garlic bulb extract and 9.4 mg/ml for garlic clove powder against *C. difficile* isolates. These results corresponded with the data from the agar well diffusion screening method, where fresh garlic bulb extract produced the largest zone of inhibition followed by garlic clove powder. However, among the raw products, only cinnamon powder showed bactericidal activity against *C. difficile*. Of the 12 processed products, approximately 67% showed some inhibitory effect at their highest concentrations with the four pure compounds allicin, *trans*-cinnamaldehyde, menthol and zingerone showing the greatest activity. No bactericidal effect was observed from any of the garlic preparations against *C. difficile*. Meanwhile, both cinnamon and *trans*-cinnamaldehyde (a major constituent of cinnamon) showed inhibitory and bactericidal activities against both *C. difficile* and comparator bacteria. Peppermint oil (8% v/v) also displayed both inhibitory and bactericidal effects against *C. difficile* strains. Similarly, menthol, the main ingredient of peppermint oil, showed MICs of 9.4 mg/ml and MBC values of 18.8 mg/ml against *C. difficile*. Interestingly, zingerone (in contrast with both fresh ginger rhizome extract and ginger rhizome powder) showed reasonable activity against *C. difficile* with MIC and MBC values of 0.4 and 18.8 mg/ml, respectively. The remaining products such as *Leptospermum* honeys, artichoke capsules and aloe vera gel also had some inhibitory effect within the range of concentrations used; however, the aloe vera gel contained 1% preservatives such as disodium edetate, which may have contributed to its antimicrobial activity. To control for the effect of solvent, the 20% DMSO solution was serially diluted 2-fold and tested against all the microorganisms. While 10% DMSO showed some inhibitory activity, no effect was observed with 5% DMSO or lower. Where initial tests indicated MICs or MBCs of 150 mg/ml with 20% DMSO, the test was repeated with 10% DMSO instead of 20% DMSO to remove any DMSO effect.

Table 3.3 Susceptibility of microorganisms to natural products determined by the broth microdilution method.

Treatment			Solvent	Microorganisms					
				<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 25922	<i>C. perfringens</i> ATCC 13124	<i>C. difficile</i> ATCC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 43598
Raw product									
Fresh garlic bulb % (v/v) ^a	MIC/MBC	SDW	0.8/12.5	0.8/6.3	0.8/6.3	0.8/50	0.8/50	0.8/50	0.8/50
Fresh ginger juice % (v/v) ^a	MIC/MBC	SDW	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50	>50/>50
Fresh onion bulb % (v/v) ^a	MIC/MBC	SDW	>50/>50	>50>50	25/>50	>50/>50	>50/>50	>50/>50	>50/>50
<i>Leptospermum</i> honey (MGO 514+) (A) % (w/v) ^b	MIC/MBC	SDW	8/16	16/16	16/>32	16/>32	16/>32	16/>32	16/>32
<i>Leptospermum</i> honey (MGO 263+) (B) % (w/v) ^b	MIC/MBC	SDW	16/32	32/32	32/>32	32/>32	32/>32	32/>32	32/>32
<i>Leptospermum</i> honey (MGO 263+) (C) % (w/v) ^b	MIC/MBC	SDW	32/>32	32/>32	32/>32	32/>32	32/>32	32/>32	32/>32
Garlic clove powder (mg/ml) ^c	MIC/MBC	SDW	9.4/37.5	4.7/18.8	18.8/37.5	9.4/>150	9.4/>150	9.4/>150	9.4/>150
	MIC/MBC	20% DMSO	4.7/37.5	4.7/18.8	9.4/18.8	9.4/18.8	9.4/>150	9.4/9.4	9.4/>150
Ginger rhizome powder (mg/ml) ^c	MIC/MBC	SDW	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150
	MIC/MBC	20% DMSO	75/150	150/150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150
Cinnamon root powder (mg/ml) ^c	MIC/MBC	SDW	37.5/>150	>150/>150	>150/>150	>150/>150	150/>150	>150/>150	150/>150
	MIC/MBC	20% DMSO	37.5/150	150/150	75/>150	75/75	75/75	75/150	75/75
Turmeric root powder (mg/ml) ^c	MIC/MBC	SDW	>150/>150	>150/>150	>150/>150	>150/>150	150/>150	>150/>150	>150/>150
	MIC/MBC	20% DMSO	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150

Table 3.3 (Cont.) Susceptibility of microorganisms to natural products determined by the broth microdilution method.

			Microorganisms						
Treatment		solvent	<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 25922	<i>C. perfringens</i> ATCC 13124	<i>C. difficile</i> ATCC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 43598	<i>C. difficile</i> ATCC 700057
Processed product									
Garlic tablet (mg/ml) ^c	MIC/MBC	20% DMSO	37.5/150	37.5/150	75/150	37.5/>150	37.5/>150	75/>150	37.5/>150
Ginger tablet (mg/ml) ^c	MIC/MBC	20% DMSO	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150
Turmeric tablet (mg/ml) ^c	MIC/MBC	20% DMSO	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150
Cinnamon tablet (mg/ml) ^c	MIC/MBC	20% DMSO	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150
Artichoke capsule (mg/ml) ^c	MIC/MBC	20% DMSO	150/150	150/150	150/150	75/>150	75/>150	150/>150	75/>150
Coconut oil capsule % (v/v) ^b	MIC/MBC	20% DMSO	>32/>32	>32/>32	>32/>32	>32/>32	>32/>32	>32/>32	>32/>32
Peppermint oil % (v/v) ^b	MIC/MBC	20% DMSO	8/16	16/32	8/8	8/8	8/8	8/8	8/8
Aloe vera gel % (w/v) ^b	MIC/MBC	20% DMSO	16/32	16/32	16/>32	16/>32	16/>32	16/>32	16/>32
Allicin (mg/ml) ^c	MIC/MBC	20% DMSO	18.8/>37.5	18.8/37.5	4.7/4.7	4.7/>37.5	2.3/>37.5	4.7/>37.5	4.7/>37.5
<i>trans</i> -Cinnamaldehyde % (v/v) ^b	MIC/MBC	20% DMSO	0.02/0.06	0.06/0.25	0.01/0.01	0.02/0.02	0.02/0.02	0.02/0.02	0.02/0.02
Zingerone (mg/ml) ^c	MIC/MBC	20% DMSO	9.4/37.5	9.4/9.4	9.4/18.8	9.4/9.4	9.4/18.8	9.4/9.4	9.4/9.4
Menthol (mg/ml) ^c	MIC/MBC	20% DMSO	9.4/37.5	9.4/9.4	18.8/18.8	9.4/18.8	9.4/18.8	9.4/18.8	9.4/18.8
Control									
Vancomycin (µg/ml) ^d	MIC/MBC	SDW	ND	ND	ND	ND	ND	ND	0.5/>16

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; MGO, methylglyoxal; ND, not done; Dilutions starting at ^a50, ^b32, ^c150, ^d16.

The effect of natural products against five commensal flora of the gastrointestinal tract, including *B. fragilis* NCTC 9343, *B. vulgatus* NCTC 10583, *B. thetaiotaomicron* 5018, *L. casei* Q328 and *L. casei* M2971 was also examined (Table 3.4). Lactic acid bacteria such as *Lactobacillus casei* were less susceptible to most of the natural products compared to *Bacteroides* and *C. difficile*. Most of the natural products with activity against the two *L. casei* strains showed MICs that were at least a 4-fold higher compared to the three *Bacteroides* species. Most of the products did not show any bactericidal activity against the lactic acid bacteria except for peppermint oil and the four pure compounds. For stationary-phase *C. difficile* cultures, 10% DMSO showed some inhibitory and bactericidal activity, but no effect was observed with 5% DMSO or lower.

3.2.3 Synergy testing using the checkerboard method

The drug interaction assay using the checkerboard method was carried out using two *C. difficile* strains; ATCC 700057 and NCTC 13366. Combining *trans*-cinnamaldehyde with vancomycin or metronidazole decreased the MIC values for both when tested using *C. difficile* strains in comparison with either agent individually (Figure 3.1). The combination of *trans*-cinnamaldehyde and vancomycin showed at least a 4-fold increase in the inhibitory effect of vancomycin and a 2-fold increase in the inhibitory activity of *trans*-cinnamaldehyde with most concentrations. All the FIC indices were < 0.5 when tested against *C. difficile* ATCC 700057 and ranged from 0.3750 to 0.5625 against *C. difficile* NCTC 13366 (Table 3.5). According to the results, *trans*-cinnamaldehyde enhanced the *in-vitro* activity of vancomycin against the two reference strains of *C. difficile* and based on the calculated FIC indices, *trans*-cinnamaldehyde had a possible synergistic effect with vancomycin. Moreover, when *trans*-cinnamaldehyde was combined with metronidazole, a ≥ 2 -fold decrease in the MIC values of *trans*-cinnamaldehyde and metronidazole against both *C. difficile* strains was observed, with

Table 3.4 Susceptibility of the representative commensal flora of the gastrointestinal tract to natural products.

Treatment			Microorganisms				
			<i>B. fragilis</i> NCTC 9343	<i>B. vulgatus</i> NCTC 10583	<i>B. thetaiotaomicron</i> 5018	<i>Lactobacillus casei</i> Q328	<i>L. casei</i> M2971
Raw product							
Fresh garlic bulb % (v/v) ^a	MIC/MBC	SDW	1.6/3.1	0.8/1.6	0.8/3.1	6.3/>50	6.3/>50
Fresh ginger juice % (v/v) ^a	MIC/MBC	SDW	>50/>50	50/>50	50/>50	>50/>50	>50/>50
Fresh onion bulb % (v/v) ^a	MIC/MBC	SDW	>50/>50	50/>50	>50/>50	>50/>50	>50/>50
<i>Leptospermum</i> honey (MGO 514+) (A) % (w/v) ^b	MIC/MBC	SDW	2/8	1/1	1/1	32/>32	32/>32
<i>Leptospermum</i> honey (MGO 263+) (B) % (w/v) ^b	MIC/MBC	SDW	2/8	1/1	1/1	32/>32	32/>32
<i>Leptospermum</i> honey (MGO 263+) (C) % (w/v) ^b	MIC/MBC	SDW	2/8	1/1	1/1	32/>32	32/>32
Garlic clove powder (mg/ml) ^c	MIC/MBC	SDW	9.4/18.8	9.4/18.8	9.4/18.8	37.5/>150	37.5/>150
Ginger rhizome powder (mg/ml) ^c	MIC/MBC	20% DMSO	150/>150	150/150	150/>150	>150/>150	>150/>150
Cinnamon root powder (mg/ml) ^c	MIC/MBC	20% DMSO	75/150	75/75	75/150	150/>150	150/>150
Turmeric root powder (mg/ml) ^c	MIC/MBC	20% DMSO	150/>150	150/150	150/>150	>150/>150	>150/>150
Processed product							
Garlic tablet (mg/ml) ^c	MIC/MBC	20% DMSO	75/150	75/150	75/150	150/>150	150/>150
Ginger tablet (mg/ml) ^c	MIC/MBC	20% DMSO	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150
Turmeric tablet (mg/ml) ^c	MIC/MBC	20% DMSO	>150/>150	>150/>150	>150/>150	>150/>150	>150/>150

Table 3.4 (Cont.) Susceptibility of the representative commensal flora of the gastrointestinal tract to natural products.

Treatment			Microorganisms				
			<i>B. fragilis</i> NCTC 9343	<i>B. vulgatus</i> NCTC 10583	<i>B. thetaiotaomicron</i> 5018	<i>Lactobacillus casei</i> Q328	<i>L. casei</i> M2971
Processed product							
Cinnamon tablet (mg/ml)^c	MIC/MBC	20% DMSO	>150/>150	150/>150	>150/>150	>150/>150	>150/>150
Artichoke tablet (mg/ml)^c	MIC/MBC	20% DMSO	75/150	75/150	75/150	>150/>150	>150/>150
Coconut oil capsule % (v/v)^b	MIC/MBC	20% DMSO	>32/>32	>32/>32	>32/>32	>32/>32	>32/>32
Peppermint oil % (v/v)^b	MIC/MBC	20% DMSO	1/1	4/4	1/1	4/8	8/8
Aloe Vera gel % (w/v)^b	MIC/MBC	20% DMSO	16/32	16/16	32/32	32/>32	32/>32
Allicin (mg/ml)^c	MIC/MBC	20% DMSO	9.4/18.8	9.4/18.8	9.4/9.4	37.5/>37.5	37.5/>37.5
<i>trans</i>-Cinnamaldehyde % (v/v)^b	MIC/MBC	20% DMSO	0.02/0.1	0.02/0.03	0.02/0.03	1/2	2/2
Zingerone (mg/ml) ^c	MIC/MBC	20% DMSO	4.7/18.8	4.7/9.4	4.7/9.4	18.8/18.8	18.8/37.5
Menthol (mg/ml)^c	MIC/MBC	20% DMSO	4.7/18.8	4.7/9.4	4.7/18.8	18.8/18.8	18.8/37.5

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; MGO, methylglyoxal. Dilutions starting at ^a50, ^b32, ^c150.

Table 3.5 MIC and FIC index values of *trans*-cinnamaldehyde and antimicrobials against *C. difficile* strains.

Organism	Metronidazole			Vancomycin		
	<i>trans</i> -Cinn	MET	FIC	<i>trans</i> -Cinn	VAN	FIC
<i>C. difficile</i> ATCC 700057						
<i>trans</i> -Cinnamaldehyde	0.0313	-	-	0.0313	-	-
Antibiotic alone	-	0.5000	-	-	2.0000	-
Combination 1	0.0039	0.250	0.6250	0.0009	0.5000	0.2813
Combination 2	0.0078	0.1250	0.5000	0.0078	0.2500	0.3750
Combination 3	0.0156	0.0313	0.5624	0.0156	0.2500	0.3750
<i>C. difficile</i> NCTC 13366						
<i>trans</i> -Cinnamaldehyde	0.0313	-	-	0.0156	-	-
Antibiotic alone	-	4.0000	-	-	1.0000	-
Combination 1	0.0039	2.0000	0.6250	0.0019	0.250	0.3750
Combination 2	0.0078	1.0000	0.5000	0.0039	0.1250	0.3750
Combination 3	0.0156	0.5000	0.6250	0.0078	0.0625	0.5625

MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration; *trans*-Cinn, *trans*-cinnamaldehyde; MET, metronidazole; VAN, vancomycin

Figure 3.1 Isobolograms representing the effect of *trans*-cinnamaldehyde on antimicrobials against *C. difficile*. a) *trans*-cinnamaldehyde and vancomycin against *C. difficile* ATCC 700057, b) *trans*-cinnamaldehyde and vancomycin against *C. difficile* NCTC 13366, c) *trans*-cinnamaldehyde and metronidazole against *C. difficile* ATCC 700057, d) *trans*-cinnamaldehyde and metronidazole against *C. difficile* NCTC 13366.

FIC indices ranging from 0.5 to 0.625 (Table 3.5). Therefore, a partial synergistic effect of *trans*-cinnamaldehyde with metronidazole might be considered. On the other hand, when the remaining products were combined with either vancomycin or metronidazole, no effect on the MIC values relative to each agent alone was observed. Hence, negligible interactions were observed for the remaining natural products against *C. difficile* isolates.

3.3 DISCUSSION

Antimicrobial treatment failure and the increasing frequency of hyper-virulent *C. difficile* strains have driven researchers to look for new therapeutic agents, such as naturally occurring compounds. In this study, the antimicrobial activity of several natural products was investigated against *C. difficile* and comparator microorganisms. Garlic was one product that demonstrated substantial activity against *C. difficile*. In general, garlic has a broad spectrum of antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria¹⁴². Allicin as the main active compound in garlic is thought to be largely responsible for its antibacterial activity^{142,144,200}.

3.3.1 Preliminary screening of natural products for antimicrobial activity

As shown by the agar well diffusion assay, which is a well-known bioactivity screening assay, the highest activity was observed from the fresh garlic bulb extract. Also, all garlic products showed good solubility in both SDW and 20% DMSO, making garlic a suitable and feasible agent to investigate. The agar well diffusion assay is a simple and quick method to generate results, especially when screening large numbers of agents^{201,202}. However, there are limitations associated with this technique. For instance, there are factors other than the susceptibility of the microorganism that may affect the size of the inhibition zone²⁰¹ such as the ability of products to diffuse through the agar medium, composition of the agar medium and incubation conditions^{201,203}. Even though this

method may not be as sensitive as some other antimicrobial activity assays, it is still used as a quick screening technique by many researchers, allowing the comparison of results with those obtained by other methods ²⁰³.

3.3.2 Broth microdilution

The broth microdilution technique provides more quantitative results than the agar diffusion method and can distinguish between inhibitory and bactericidal activity. Although this technique is used extensively, limitations such as poor growth of some anaerobic species are still an issue. However, this study followed the methods recommended by CLSI ^{164,165}, and thus consistent and reliable results were generated.

3.3.2.1 Susceptibility of *C. difficile* isolates to natural products

The antimicrobial activity of natural products against *C. difficile* has been investigated in several previous studies. For instance, a study by Hammond and Donkor investigated the effect of Woundcare™ 18+ active manuka honey from Comvita UK against three *C. difficile* strains, including a reference strain (ATCC 9689) and two clinical isolates of RTs 027 and 106 ¹¹⁶. The study was carried out using both agar well diffusion and broth microdilution techniques. In their study, the zone of inhibition for 50% v/v manuka honey solution ranged from 14.2 to 14.5 mm for the three strains of *C. difficile* after 2 days of incubation ¹¹⁶. In addition, the manuka honey showed the same MIC and MBC values of 6.25 % v/v against the three *C. difficile* strains, suggesting a bactericidal activity ¹¹⁶. However, in the present study, MICs for the three *Leptospermum* honeys against the four *C. difficile* strains were 2- to 4-fold higher than those obtained in the Hammond and Donkor study and no bactericidal activity was observed at the highest concentration of honey solutions used. This could be due to the use of different strains of *C. difficile* in each study. Moreover, different samples of honey were tested, and the activity of honey

can vary significantly depending on several factors; including the spatial and temporal variation in sources of nectar ¹⁶¹.

Another study by Shilling *et al.*, investigated the *in-vitro* activity of virgin coconut oil and its most active fatty acids on toxin A and B positive *C. difficile* ATCC 9689 ¹¹⁸. Virgin coconut oil did not show any inhibitory effect against this *C. difficile* strain, however, when the coconut oil was digested with porcine lipase, the resulting product inhibited 99.9% of the overnight growth of *C. difficile* at the concentration of 1.2% v/v ¹¹⁸. The lauric acid component, which constitutes up to 48% of the saturated fat in coconut oil, at a concentration of only 250 µM inhibited 90% of the bacterial growth compared to the controls (2% DMSO and 1% ethyl alcohol) ¹¹⁸. In the present study, no antimicrobial activity was observed for undigested virgin coconut oil against any of the tested microorganisms using either agar well diffusion or broth microdilution technique. Therefore, our findings correlated with the results of the previous study ¹¹⁸.

Another study has reported *in-vitro* antimicrobial activity of pomegranate extract against *C. difficile* ¹¹⁹. The antimicrobial activity of pomegranate extracts was investigated against 29 clinical *C. difficile* isolates using an agar dilution method ¹¹⁹. Also, a Folin-Ciocalteu colorimetric method was used to determine the total phenolic content of pomegranate extract and the final concentrations of 6.25 to 400 µg/ml gallic acid equivalent were achieved. The MIC values for the extracts ranged from 12.5 to 25 mg/ml for all the strains, which suggested the possible efficacy of pomegranate in the treatment of CDI ¹¹⁹. The antimicrobial activity of black seed oil (*Nigella sativa*) and myrrh (*Commiphora myrrha*) water extracts were also investigated against two *C. difficile* strains using an agar well diffusion assay ¹²⁰. Both black seed oil (2% v/v) and myrrh water extracts inhibited *C. difficile* growth *in-vitro* showing zones of inhibition ≥ 15 mm

¹²⁰.

3.3.2.2 Effect of natural products on gut microbiota

Although both the conventional antimicrobials vancomycin and metronidazole possess antibacterial activity against *C. difficile*, there is still a risk of infection recurrence following treatment ²⁰⁴. When antimicrobials are used for treatment, they may have a negative impact on the commensal gut microflora, thus enhancing the risk of recurrent infection ²⁰⁴. Several studies have reported the effect of antimicrobials such as vancomycin and metronidazole on intestinal flora ²⁰⁴⁻²⁰⁶. Edlund *et al.*, assessed the effect of oral vancomycin on the intestinal flora of patients who had previously received antimicrobial therapy ²⁰⁵. Bifidobacteria and *Bacteroides* spp. were eliminated during vancomycin administration, which subsequently resulted in the development of vancomycin-resistant enterococci, *Pediococcus* species and lactobacilli ²⁰⁵.

In the present study, both *L. casei* strains were less susceptible to most of the natural therapeutic agents compared to *C. difficile* and *Bacteroides*, as less impact on *L. casei* was observed *in-vitro*. This is a potential advantage to using natural products since they demonstrated activity against *C. difficile* at concentrations lower than those affecting *Lactobacillus* spp. and may be considered as a supplementary therapeutic option that is less disruptive to the commensal flora. *L. casei* is a commercial probiotic strain ²⁰⁷ and the use of anti-*C. difficile* agents with less activity against this commensal organism may be beneficial for treatment. Previously, the benefit of using garlic as a health supplement against various pathogens including clostridia in the gut has been suggested, as it would not adversely impact the commensal flora of the gastrointestinal tract ²⁰⁸. In that study, a rapid killing of *Bacteroides* and bifidobacteria by garlic was observed while *L. casei* strains showed less susceptibility ²⁰⁸.

3.3.3 Drug interactions

Vancomycin is a glycopeptide that can inhibit cell wall synthesis in susceptible microorganisms by preventing the polymerization of the phosphodisaccharide-pentapeptide-lipid complex ²⁰⁹. However, additional modes of action for vancomycin have been suggested in some studies, such as the alteration of cell membrane permeability and selective inhibition of RNA synthesis ^{209,210}. Alternatively, metronidazole inhibits DNA synthesis by microbial cells and causes the breakage of DNA strands through oxidation leading to eventual cell death ²¹¹.

trans-Cinnamaldehyde is an essential oil component of cinnamon and it possesses antimicrobial activity against a range of pathogens ²¹². It has been postulated that the hydrophobic nature of many essential oil components allows the components to penetrate microbial membranes and alter their integrity, leading to the leakage of cell constituents ²¹³⁻²¹⁵. Hydrophobic molecules can penetrate the cell wall of Gram-positive bacteria and affect both cell wall and cytoplasmic content. In contrast, the outer membrane of Gram-negative bacteria is not completely impermeable to hydrophobic molecules ²¹³, and only some are able to pass through porins in the outer membrane ²¹⁴.

Essential oils and their components can have more than one target for their activity ^{202,216}. For instance, *trans*-cinnamaldehyde can prevent the growth of *E. coli* by gaining access to the periplasm and deeper regions of the bacterial cell without altering the outer membrane ²¹⁷. In a study by Domadia *et al.*, *trans*-cinnamaldehyde inhibited cell division in *B. cereus* ²¹⁸. Different modes of action have been suggested for *trans*-cinnamaldehyde, including the inhibition of cell wall synthesis similar to vancomycin. This could explain how *trans*-cinnamaldehyde may enhance the activity of this antimicrobial. In addition, if the inhibition of cell division is considered another possible mechanism of action for *trans*-cinnamaldehyde, it would clarify the partial enhancement of activity observed with *trans*-cinnamaldehyde and metronidazole, as this antimicrobial acts by inhibiting DNA

synthesis. The remaining products did not have any effect on the activity of either antimicrobial, therefore the consumption of those natural products with activity against *C. difficile* in conjunction with antimicrobials may be considered. Nevertheless, the consumption of a high dose of some of these compounds, such as *trans*-cinnamaldehyde, may be toxic to humans and precautions regarding the safe use of these products should be followed.

3.3.4 Safe use of natural products

Side effects occur with many conventional drugs, and also with some natural products, therefore they should not always be assumed to be safe ²¹⁹. Some of these adverse events are due to the inherent toxicity of the plant extract, the use of the wrong species of plant, incorrect dosing, interactions with other medications and contamination with hazardous substances ²²⁰. It is important to identify the risks associated with the use of herbal medicine through pre-clinical and clinical toxicological evaluation ²²¹. This would identify the toxic compounds which can be discarded or modified to provide a safer alternative ²¹⁹. Dosage adjustment and modification of certain chemical structures may also enhance their tolerability. It is important to recognize that the activity of natural products may vary between different samples of the same plant and varies depending on the plant species they are derived from. Further studies on the chemical composition of these products are of importance and until such studies are conducted, investigations of “natural products” should be interpreted with caution.

In this and subsequent chapters, products showing activity at concentrations that are safe for human consumption were considered worthy of further investigation. Although no chemical analysis of crude extracts was performed, the pure active components in some, such as allicin, *trans*-cinnamaldehyde, menthol and zingerone, were tested, and in some cases showed activity.

3.4 CONCLUSIONS

This chapter highlights the antimicrobial activity of several natural products against *C. difficile in-vitro*, while showing less impact on members of the gut commensal flora such as *L. casei*. This suggests that the active natural products may be worthwhile to further investigate for the treatment of CDI. Additionally, as *trans*-cinnamaldehyde at low concentrations enhanced the antimicrobial activity of conventional antimicrobials *in-vitro*, combination therapy with this compound and conventional antimicrobials may improve the prognosis for this infection.

Chapter 4

Effects of natural products on several stages of the spore cycle of *C. difficile in-vitro*

The following publication has arisen from this chapter:

Roshan N, Riley TV, Hammer KA. Effects of natural products on several stages of the spore cycle of *Clostridium difficile in-vitro*. *Journal of Applied Microbiology* 2018; **125**: 710-23.

4.1 INTRODUCTION

C. difficile exists in both vegetative and spore forms. Bacterial spores are structurally different from the vegetative cells, giving them an extreme resistance that enables them to survive adverse conditions ²²². Sporulation is an adaptive process that produces endospores that are naturally resistant to environmental stress ²²³. *C. difficile* spores are resistant to a wide array of physical and chemical agents ²²². When infection occurs, vegetative cells of *C. difficile* initiate a sporulation process, producing dormant endospores that persist in the host's colon, becoming a source for disease transmission and recurrence ^{224,225}. After the spores are ingested, they germinate into toxin-producing vegetative cells once they reach the conducive environment of the gut. In susceptible individuals, this process results in diarrhoeal disease ²²⁶.

During spore germination, some spore-specific characters are irreversibly lost, and there is little known about the mechanism involved in spore germination of *C. difficile* ²²⁴. *C. difficile* spores are metabolically dormant and are intrinsically resistance to all known antimicrobial therapies, the host's immune system and bleach-free disinfectants ²²⁷⁻²²⁹. Since *C. difficile* spores are the infective particle essential for dissemination and persistence of the pathogen, both sporulation and germination are critical steps in CDI. Therefore, exploring the antibacterial agents that have an effect on bacterial spores has become a focus for finding new anti-*C. difficile* therapies ²³⁰.

4.1.1 Aims

The aim of this chapter was to determine the effect of natural products on sporulation, germination and outgrowth of *C. difficile* spores. In addition, sporicidal activity was determined for those products showing bactericidal activity against vegetative cells.

4.2 RESULTS

4.2.1 Antimicrobial susceptibility testing

The susceptibility of stationary- and exponential-phase cultures of all four strains of *C. difficile* to antimicrobial agents is shown in Table 3.3 and 4.1, respectively. All natural products with the exception of fresh ginger rhizome extract showed activity against all four mid-exponential phase *C. difficile* strains. *trans*-Cinnamaldehyde was the most active compound with MICs as low as 0.02% v/v against all four *C. difficile* strains. The susceptibility of stationary-phase *C. difficile* strains is described in the previous chapter. Stationary-phase *C. difficile* were less susceptible to the products compared to mid-exponential phase cultures and had higher MIC values. However, *trans*-cinnamaldehyde was still the most active compound against the stationary phase culture (MICs = 0.02% v/v). For the DMSO solvent control, the 10% and 5% DMSO showed inhibitory activity against mid-exponential phase *C. difficile* strains, but no effect was observed with 2.5% DMSO or lower.

4.2.2 Sporulation assays

The effect of $0.5 \times \text{MIC}$ of each natural product on the sporulation of *C. difficile* NCTC 13366 is shown in Table 4.2. Cultures treated with $0.5 \times \text{MIC}$ of each product were compared to untreated controls by measuring OD₆₀₀ to ensure that $0.5 \times \text{MIC}$ did not significantly inhibit growth and that similar growth was achieved. Growth was not significantly affected by $0.5 \times \text{MIC}$. For each treated sample, the proportion of spores was compared with untreated and solvent controls (SDW and DMSO). Fresh ginger rhizome extract (50% v/v), fresh onion bulb extract (12.5% v/v) and coconut oil (16% v/v) at $0.5 \times \text{MIC}$ significantly reduced spore formation by *C. difficile* NCTC 13366 in both conventional spore recovery and microscopy assays after 48 and 96 h incubation ($P < 0.001$) (Table 4.2; Figure 4.1). The remaining treatments had no significant effect on

Table 4.1 MICs of natural products against exponential-phase cultures of *C. difficile*.

Treatment	Solvent	Microorganisms				Concentration used for germination and outgrowth assay ¹
		<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 43598	<i>C. difficile</i> ATCC 700057	
<u>Raw product</u>						
Fresh garlic bulb % (v/v) ^a	SDW	0.4	0.4	0.4	0.4	50
Fresh ginger rhizome % (v/v) ^a	SDW	>50	>50	>50	>50	50
Fresh onion bulb % (v/v) ^a	SDW	25	25	25	25	50, 25, 12.5 ²
<i>Leptospermum</i> honey (MGO 514+) (A) % (w/v) ^b	SDW	4	4	4	4	32, 16, 8 ²
<i>Leptospermum</i> honey (MGO 263+) (B) % (w/v) ^b	SDW	4	4	4	4	32, 16, 8 ²
<i>Leptospermum</i> honey (MGO 263+) (C) % (w/v) ^b	SDW	8	8	8	8	32, 16, 8 ²
Garlic clove powder (mg/ml) ^c	20% DMSO	2.3	2.3	2.3	2.3	75
Ginger rhizome powder(mg/ml) ^c	20% DMSO	75	37.5	37.5	37.5	75
Cinnamon root powder (mg/ml) ^c	20% DMSO	18.8	9.4	9.4	9.4	75
Turmeric root powder (mg/ml) ^c	20% DMSO	75	37.5	37.5	37.5	75
<u>Processed products</u>						
Garlic tablet (mg/ml) ^c	20% DMSO	37.5	37.5	37.5	37.5	75
Ginger tablet (mg/ml) ^c	20% DMSO	75	75	75	75	75
Cinnamon tablet (mg/ml) ^c	20% DMSO	18.8	18.8	18.8	18.8	75
Turmeric tablet (mg/ml) ^c	20% DMSO	75	37.5	75	75	75
Artichoke capsule (mg/ml) ^c	20% DMSO	18.8	9.4	9.4	9.4	75, 37.5, 18.8 ²
Aloe Vera gel % (w/v) ^b	20% DMSO	16	16	16	16	32
Coconut oil capsule % (v/v) ^b	20% DMSO	32	32	32	32	32
Peppermint oil % (v/v) ^b	20% DMSO	1	1	1	1	32
<u>Pure compounds</u>						
Allicin (mg/ml) ^c	20% DMSO	4.7	2.3	4.7	4.7	75, 37.5, 18.8 ²
<i>trans</i> -Cinnamaldehyde % (v/v) ^b	20% DMSO	0.02	0.02	0.02	0.02	32
Menthol (mg/ml) ^c	20% DMSO	2.3	2.3	2.3	2.3	75
Zingerone (mg/ml) ^c	20% DMSO	4.7	4.7	4.7	4.7	75
<u>Control</u>						
Fidaxomicin (µg/ml) ^d	20% DMSO	0.125	0.125	0.125	0.125	16, 8, 4 ²

MIC, minimum inhibitory concentration; SDW, sterile distilled water; MGO, methylglyoxal; ND, not done.

*Serial two-fold dilutions were performed in SDW for all the products.

***Leptospermum* honey (A), MGO 514+; *Leptospermum* honey (B), MGO 263+; *Leptospermum* honey (C), MGO 263+.Dilutions starting at ^a50, ^b32, ^c150, ^d16.¹ In some instances, concentrations could not be supra-MIC due to DMSO or not showing a MIC value in the range of concentration of treatments used.² For the products that showed an effect, the assay was repeated with two-fold and four-fold lower concentrations.

Dash means no solvent.

Table 4.2 Proportion of spores of *C. difficile* NCTC 13366 after 48 and 96 h of growth in TY broth containing $0.5 \times \text{MIC}$ of natural products.

Antimicrobial agents (concentration)	% spores $\pm$ SD			
	Conventional spore recovery assay		Microscopy	
	48 h	96 h	48 h	96 h
Fresh garlic bulb extract (0.2% v/v)	8.6 $\pm$ 0.5	17.9 $\pm$ 1.2	17.0 $\pm$ 2.8	29.4 $\pm$ 2.9
Fresh ginger rhizome extract (50% v/v)	1.6 $\pm$ 0.4*	3.2 $\pm$ 0.3*	7.0 $\pm$ 2.1*	10.5 $\pm$ 2.1*
Fresh onion bulb extract (12.5% v/v)	0.8 $\pm$ 0.1*	4.2 $\pm$ 0.3*	4.2 $\pm$ 0.4*	7.6 $\pm$ 1.1*
<i>Leptospermum</i> honey (A) (4% w/v)	7.6 $\pm$ 0.5	18.3 $\pm$ 2.0	16.9 $\pm$ 1.9	30.3 $\pm$ 2.7
<i>Leptospermum</i> honey (B) (4% w/v)	8.1 $\pm$ 2.4	17.6 $\pm$ 1.3	18.4 $\pm$ 3.8	32.0 $\pm$ 2.5
<i>Leptospermum</i> honey (C) (4% w/v)	9.5 $\pm$ 1.4	18.1 $\pm$ 1.4	17.2 $\pm$ 2.8	31.6 $\pm$ 2.1
Garlic clove powder (1.2 mg/ml)	7.9 $\pm$ 0.7	16.8 $\pm$ 1.6	19.1 $\pm$ 2.5	31.0 $\pm$ 3.0
Ginger rhizome powder (37.5 mg/ml)	7.8 $\pm$ 1.3	16.2 $\pm$ 1.7	18.3 $\pm$ 2.7	28.8 $\pm$ 3.1
Cinnamon root powder (9.4 mg/ml)	9.6 $\pm$ 0.6	18.5 $\pm$ 2.8	20.1 $\pm$ 2.3	32.8 $\pm$ 2.7
Turmeric root powder (37.5 mg/ml)	7.1 $\pm$ 0.9	18.1 $\pm$ 1.3	16.3 $\pm$ 2.3	27.1 $\pm$ 3.8
Garlic tablet (18.8 mg/ml)	7.6 $\pm$ 0.1	15.9 $\pm$ 0.7	20.3 $\pm$ 2.1	30.4 $\pm$ 2.8
Ginger tablet (37.5 mg/ml)	8.4 $\pm$ 0.6	14.0 $\pm$ 2.5	19.5 $\pm$ 1.5	28.9 $\pm$ 3.4
Cinnamon tablet (9.4 mg/ml)	9.7 $\pm$ 0.6	15.1 $\pm$ 1.5	18.4 $\pm$ 3.1	36.0 $\pm$ 2.5
Turmeric tablet (37.5 mg/ml)	7.1 $\pm$ 0.9	15.1 $\pm$ 1.3	18.5 $\pm$ 1.9	34.3 $\pm$ 3.4
Artichoke capsule (9.4 mg/ml)	7.4 $\pm$ 1.6	16.4 $\pm$ 1.2	16.7 $\pm$ 1.1	32.0 $\pm$ 3.6
Aloe vera (8% w/v)	9.0 $\pm$ 0.4	13.5 $\pm$ 2.7	17.6 $\pm$ 3.2	34.1 $\pm$ 3.8
Coconut oil (16% v/v)	2.8 $\pm$ 0.7*	4.2 $\pm$ 0.7*	4.1 $\pm$ 1.3*	6.4 $\pm$ 1.0*
Peppermint oil (1% v/v)	9.6 $\pm$ 0.7	17.3 $\pm$ 2.4	16.1 $\pm$ 1.9	30.3 $\pm$ 3.9
Allicin (2.3 mg/ml)	8.0 $\pm$ 0.9	15.5 $\pm$ 2.4	15.9 $\pm$ 2.6	27.1 $\pm$ 3.5
<i>trans</i> -Cinnamaldehyde (0.001% v/v)	8.3 $\pm$ 0.4	15.7 $\pm$ 1.3	17.4 $\pm$ 3.3	30.5 $\pm$ 2.6
Menthol (2.3 mg/ml)	7.7 $\pm$ 0.2	13.5 $\pm$ 2.8	15.7 $\pm$ 3.5	32.7 $\pm$ 3.4
Zingerone (4.7 mg/ml)	7.8 $\pm$ 0.3	16.5 $\pm$ 3.2	15.7 $\pm$ 3.6	28.4 $\pm$ 2.8
Fidaxomicin (0.125 mg/ml)	0.6 $\pm$ 0.3*	2.5 $\pm$ 0.4*	1.2 $\pm$ 0.8*	3.0 $\pm$ 0.7*
Untreated control (SDW)	8.8 $\pm$ 0.9	17.4 $\pm$ 1.5	19.4 $\pm$ 2.7	33.8 $\pm$ 1.9
Untreated control (DMSO) (2.5% v/v)	8.0 $\pm$ 0.9	19.2 $\pm$ 1.9	16.2 $\pm$ 2.9	32.3 $\pm$ 3.6

SD, standard deviation; h, hour; SDW, sterile distilled water; DMSO, dimethyl sulfoxide. *Leptospermum* honey (A), MGO 514+; *Leptospermum* honey (B), MGO 263+; *Leptospermum* honey (C), MGO 263+. Differ significantly from the untreated controls (SDW and DMSO): * = $P < 0.001$

Figure 4.1 The percentage of spores after 48 h and 96 h growth in liquid TY broth containing $0.5 \times \text{MIC}$ of natural products or antimicrobials determined by conventional spore recovery and microscopy. Concentrations of agents used in the assay: Fresh onion bulb extract (12.5% v/v); Fresh ginger rhizome extract (50% v/v); Coconut oil (16% v/v); Fidaxomicin (0.06 $\mu\text{g/ml}$) for *C. difficile* ATCC 700057, ATCC 43598 and R44116; Fidaxomicin (0.125 $\mu\text{g/ml}$) for *C. difficile* NCTC 1366; DMSO (2.5% v/v). ■ = $P < 0.05$, ♦ = $P < 0.01$, * = $P < 0.001$.

the proportion of spores compared to untreated controls.

For *C. difficile* NCTC 13366, fresh ginger rhizome extract, fresh onion bulb extract and coconut oil were also tested at $0.25 \times \text{MIC}$. In addition, these three products, as well as the controls, were assessed against the three remaining *C. difficile* strains ATCC 700057, ATCC 43598 and R11446 at both $0.5 \times \text{MIC}$ and $0.25 \times \text{MIC}$ (Figures 4.1 and 4.2).

These three treatments at $0.5 \times \text{MIC}$ significantly inhibited spore formation in all four *C. difficile* strains by 50 to 90 % ($P \leq 0.042$). The only exception was fresh ginger rhizome extract against *C. difficile* ATCC 43598, which did not significantly reduce spore formation after either 48 or 96 h and had no inhibitory effect at 96 h for *C. difficile* ATCC 700057 ($P > 0.05$). Spore formation for all four *C. difficile* strains was significantly inhibited by fresh onion bulb extract at $0.25 \times \text{MIC}$ (6.3% v/v) compared to the untreated and solvent controls ($P \leq 0.041$). Coconut oil at $0.25 \times \text{MIC}$ (8% v/v) also inhibited spore formation by most strains except for *C. difficile* strains ATCC 43598 at 96 h ($P \leq 0.045$). Fresh ginger rhizome extract at 25% v/v showed inhibition of spore formation for the three *C. difficile* strains NCTC 13366, ATCC 700057 and R11446 only after 48 h ($P \leq 0.019$).

4.2.3 Spore germination and outgrowth assay

Spore germination was unaffected when *C. difficile* was exposed to products at concentrations that were largely supra-inhibitory for stationary-phase *C. difficile* (Figure 4.3A). Spore suspensions showed a decrease in OD_{600} until ~ 15 min to 2 h (depending on the strain), which indicated spore germination. For most spore suspensions containing treatments, an increase in OD_{600} was observed after ~ 20 min, correlating with the conversion of dormant spores to germinated spores and the commencement of vegetative outgrowth. Treatment with artichoke extract (75 mg/ml), fresh onion bulb extract

Figure 4.2 The percentage of spores after 48 h and 96 h growth in liquid TY broth containing $0.25 \times \text{MIC}$ of natural products or antimicrobials determined by conventional spore recovery and microscopy. Concentrations of agents used in the assay: Fresh onion bulb extract (6.25% v/v); Fresh ginger rhizome extract (25% v/v); Coconut oil (8% v/v); Fidaxomicin (0.03 $\mu\text{g/ml}$) for *C. difficile* ATCC 700057, ATCC 43598 and R44116; Fidaxomicin (0.06 $\mu\text{g/ml}$) for *C. difficile* NCTC 1366; DMSO (2.5% v/v). ■ = $P < 0.05$, ♦ = $P < 0.01$, * = $P < 0.001$.

Figure 4.3A Effect of natural products on germination and outgrowth of *C. difficile* spores. Concentration of agents used in the assay: DMSO (2.5% v/v), Fidaxomicin (16 μ g/ml), Artichoke (75 mg/ml), Fresh onion bulb extract (50% v/v), *Leptospermum* honeys A, B and C (32% w/v), Allicin (75 mg/ml).

(50% v/v), three *Leptospermum* honeys (32% v/v) and allicin (75 mg/ml) resulted in no significant increase in absorbance from 7 h to 24 h ($P \geq 0.691$) for spore suspensions of any strain and absorbance at 24 h differed significantly from the untreated controls ($P \leq 0.05$). The experiment was repeated with 2-fold and 4-fold lower concentrations of these six products and fidaxomicin. Similar results were found at lower concentrations for the artichoke extract and the three *Leptospermum* honeys (Figures 4.3B and 4.3C). In contrast, fresh onion bulb extract at a 4-fold lower concentration (12.5% v/v) showed a significantly higher absorbance (indicating outgrowth) at 24 h compared to 7 h ($P \leq 0.025$). Allicin at both 2-fold and 4-fold lower concentrations showed a significant outgrowth at 24 h compared to 7 h ($P \leq 0.029$). Therefore, in the presence of certain concentrations of these treatments, the late stage of *C. difficile* outgrowth remained constant and suppressed. In the presence of the remaining treatments, including controls (SDW and DMSO), a significant increase in outgrowth from 7 h to 24 h for all four *C. difficile* strains was observed ($P \leq 0.05$) (Supplementary Tables 1-4, Appendix I). Fidaxomicin and vancomycin at supra-inhibitory concentrations have been previously reported to inhibit the outgrowth of *C. difficile* spores. The antimicrobial controls, fidaxomicin and vancomycin, did not inhibit the initiation of outgrowth, but outgrowth continued at reduced rates and for some strains remained low until approximately 5 h, followed by a non-significant decrease in outgrowth from 7 h to 24 h ($P \geq 0.078$). The absorbance at 24 h for these antimicrobial controls was significantly lower than the untreated controls ($P \leq 0.001$).

4.2.4 Sporicidal activity of natural products

Among the five products tested for sporicidal activity against *C. difficile*, peppermint oil and *trans*-cinnamaldehyde exhibited some level of activity compared to the untreated controls (Tables 4.3A and 4.3B). Based on the initial testing, both peppermint oil and

Figure 4.3B Effect of natural products on germination and outgrowth of *C. difficile* spores.

Concentration of agents used in the assay: DMSO (2.5% v/v), Fidaxomicin (8 μ g/ml), Artichoke (37.5 mg/ml), Fresh onion bulb extract (25% v/v), *Leptospermum* honeys A, B and C (16% w/v), Allicin (37.5 mg/ml).

Figure 4.3C Effect of natural products on germination and outgrowth of *C. difficile* spores.

Concentration of agents used in the assay: DMSO (2.5% v/v), Fidaxomicin (4 μ g/ml), Artichoke (18.8 mg/ml), Fresh Onion bulb extract (12.5% v/v), *Leptospermum* honeys A, B and C (8% w/v), Allicin (18.8 mg/ml).

Table 4.3A Sporicidal activity of selected natural products against *C. difficile* isolates.

Antimicrobial agent (concentration)	Incubation time	Log reduction in viable spores			
		<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 43598	<i>C. difficile</i> ATCC 700057
Cinnamon root powder (75 mg/ml)	24 h	0.14 ± 0.08	0.08 ± 0.10	0.12 ± 0.06	0.06 ± 0.04
	48 h	0.21 ± 0.08	0.13 ± 0.08	0.23 ± 0.04	0.16 ± 0.07
	7 d	0.13 ± 0.09	0.17 ± 0.09	0.32 ± 0.14	0.12 ± 0.10
Peppermint oil (32% v/v)	24 h	1.22 ± 0.42***	0.99 ± 0.11***	1.19 ± 0.20***	1.2 ± 0.23***
	48 h	1.72 ± 0.12***	1.20 ± 0.21***	0.98 ± 0.28**	2.00 ± 0.28***
	7 d	1.98 ± 0.37***	1.86 ± 0.40***	1.72 ± 0.31***	1.78 ± 0.48***
<i>trans</i> -Cinnamaldehyde (32% v/v)	24 h	1.09 ± 0.34***	1.10 ± 0.26***	0.92 ± 0.49**	1.22 ± 0.08***
	48 h	1.75 ± 0.23***	1.13 ± 0.41***	1.36 ± 0.37***	1.87 ± 0.34***
	7 d	2.25 ± 0.12***	2.22 ± 0.26***	1.99 ± 0.27***	2.17 ± 0.43***
Menthol (75 mg/ml)	24 h	0.18 ± 0.16	0.23 ± 0.10	0.22 ± 0.13	0.20 ± 0.07
	48 h	0.46 ± 0.16	0.50 ± 0.17	0.40 ± 0.20	0.49 ± 0.27
	7 d	0.48 ± 0.09	0.36 ± 0.14	0.48 ± 0.08	0.57 ± 0.05
Zingerone (75 mg/ml)	24 h	0.20 ± 0.17	0.18 ± 0.03	0.24 ± 0.09	0.33 ± 0.11
	48 h	0.27 ± 0.19	0.23 ± 0.18	0.37 ± 0.15	0.19 ± 0.08
	7 d	0.53 ± 0.26	0.48 ± 0.11	0.46 ± 0.07	0.58 ± 0.25
SDW	24 h	0.08 ± 0.11	0.05 ± 0.06	0.09 ± 0.02	0.10 ± 0.09
	48 h	0.12 ± 0.07	0.07 ± 0.02	0.08 ± 0.10	0.09 ± 0.01
	7 d	0.12 ± 0.08	0.10 ± 0.05	0.07 ± 0.04	0.08 ± 0.02
DMSO (2.5% v/v)	24 h	0.07 ± 0.07	0.07 ± 0.05	0.03 ± 0.12	0.09 ± 0.03
	48 h	0.08 ± 0.05	0.05 ± 0.02	0.06 ± 0.07	0.06 ± 0.03
	7 d	0.15 ± 0.08	0.13 ± 0.08	0.13 ± 0.06	0.06 ± 0.01

SDW, sterile distilled water; DMSO, dimethyl sulfoxide; h, hour; d, day.

Statistical significance: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to the untreated controls (SDW and DMSO).

Table 4.3B Sporicidal activity of selected natural products against *C. difficile* isolates.

Microorganisms	Incubation time	Log reduction in viable spores											
		Peppermint oil (% v/v)					<i>trans</i> -Cinnamaldehyde (% v/v)					SDW	DMSO (% v/v)
		16	8	4	2	1	16	8	4	2	1		2.5
<i>C. difficile</i> NCTC 13366	24 h	1.19 ± 0.12***	1.23 ± 0.05***	1.14 ± 0.08***	0.61 ± 0.10	0.51 ± 0.11	1.12 ± 0.30***	1.00 ± 0.11***	0.72 ± 0.09**	0.68 ± 0.08*	0.69 ± 0.05*	0.08 ± 0.11	0.07 ± 0.07
	48 h	1.67 ± 0.54***	1.18 ± 0.16***	1.17 ± 0.05***	1.05 ± 0.09***	0.80 ± 0.10	1.75 ± 0.41***	1.64 ± 0.21***	1.11 ± 0.10***	1.11 ± 0.23***	0.80 ± 0.23*	0.12 ± 0.07	0.08 ± 0.05
	7 d	1.92 ± 0.22***	1.76 ± 0.23***	1.26 ± 0.29***	1.21 ± 0.23***	0.84 ± 0.14*	2.10 ± 0.21***	1.80 ± 0.26***	1.46 ± 0.28***	1.34 ± 0.12***	0.80 ± 0.31*	0.12 ± 0.08	0.15 ± 0.06
<i>C. difficile</i> ATCC 700057	24 h	1.21 ± 0.18***	1.07 ± 0.07***	0.94 ± 0.18***	0.63 ± 0.25*	0.57 ± 0.19	1.14 ± 0.24***	1.13 ± 0.21***	1.00 ± 0.13***	0.89 ± 0.26***	0.74 ± 0.11**	0.10 ± 0.09	0.09 ± 0.03
	48 h	1.72 ± 0.028***	1.55 ± 0.48***	1.68 ± 0.29***	1.25 ± 0.38***	0.88 ± 0.44	1.84 ± 0.24***	1.32 ± 0.23***	1.33 ± 0.29***	1.09 ± 0.23**	0.93 ± 0.18*	0.09 ± 0.01	0.06 ± 0.03
	7 d	1.94 ± 0.47***	1.67 ± 0.36***	1.27 ± 0.10**	1.43 ± 0.50***	1.09 ± 0.36*	2.15 ± 0.34***	1.70 ± 0.17***	1.48 ± 0.27***	0.98 ± 0.11*	0.99 ± 0.09*	0.08 ± 0.02	0.06 ± 0.01

SDW, sterile distilled water; DMSO, dimethyl sulfoxide; h, hour; d, day.

Statistical significance: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to untreated controls (SDW and DMSO).

trans-cinnamaldehyde at high concentration of 32% v/v showed significant sporicidal activity at all three exposure times (24 h, 48 h and 1 week) in comparison to the untreated controls ($P < 0.01$). Peppermint oil and *trans*-cinnamaldehyde were further tested at lower concentrations against the two strains NCTC 13366 and ATCC 700057. For *trans*-cinnamaldehyde, the $\log_{10}$ reduction values ranged from 0.68 to 2.22, with significant $\log_{10}$ reductions of 0.68 and 0.74 for both strains, NCTC 13366 and ATCC 700057 observed with *trans*-cinnamaldehyde (1% v/v) after 24 h of incubation ($P \leq 0.016$). On the other hand, peppermint oil treatment resulted in $\log_{10}$ reduction values ranging from 0.51 to 2.0. After 24 h of incubation with peppermint oil (4% v/v), $\log_{10}$ reduction values against strains NCTC 13366 and ATCC 700057 were 1.14 and 0.94 respectively ($P < 0.001$) (Table 4.8). In general, a long exposure time and a high concentration of product were required to achieve substantial sporicidal activity.

4.3 DISCUSSION

4.3.1 Inhibition of spore formation by several natural products

This chapter aimed to investigate the impact of natural products on different stages of the spore cycle of *C. difficile in-vitro*. Based on the sporulation assay, both fresh onion bulb extract and coconut oil, and to a lesser extent fresh ginger rhizome extract, inhibited the formation of spores by *C. difficile*; however, the effect varied across strains. Similar trends were observed by both, the conventional spore recovery assay and by microscopy. A control assessing the viability of vegetative cells was included in each sporulation experiment, thus the possibility that the inhibition of sporulation may have been due to a growth defect or death of vegetative cells could be eliminated. In general, microscopy showed a higher number of spores compared to the conventional spore recovery method. This could be due to the non-viability of some spores or differences in methodology, nonetheless, similar trends were observed by both techniques.

When *C. difficile* vegetative cells are exposed to certain stress factors such as nutrient deprivation, they initiate the sporulation process resulting in substantial production of dormant spores to survive in harsh environments^{231,232}. The inhibitory effects of antimicrobials on sporulation by *C. difficile* have been investigated previously in several studies^{168,233}. Garneau *et al.* determined the impact of sub-MIC concentrations ($\leq 0.5 \times$ MIC) of five antimicrobials on 10 isolates of *C. difficile in-vitro*¹⁶⁸. Both tigecycline and piperacillin/tazobactam reduced spore formation by several *C. difficile* isolates, while neither metronidazole nor vancomycin showed a significant effect on sporulation. Ciprofloxacin also reduced spore formation, but mainly for *C. difficile* RT 027 isolates¹⁶⁸.

Another study showed that fidaxomicin at a sub-inhibitory concentration inhibited spore production by approximately 50% for *C. difficile* strain ATCC 4355 and a ribotype 014 isolate²³³. In the current study, fidaxomicin at supra-inhibitory concentrations reduced the transcription of sporulation-specific mRNAs. In our study, fidaxomicin was used as a positive control for outgrowth, and showed inhibition of spore formation similar to that reported earlier²³³. It was suggested that fidaxomicin inhibits spore formation of *C. difficile* possibly by blocking the *spoIIR* and *spoIIID* mRNAs expressed during sporulation process²³³. Natural products may also exert an inhibitory effect on sporulation through similar mechanisms.

Previously, carvacrol at sub-MIC levels significantly reduced *C. difficile* sporulation as well as down-regulation of the genes involved in spore production ($P < 0.05$)²³⁴. Additionally, spore outgrowth was completely inhibited at the MIC of carvacrol²³⁴. However, only a few studies have reported the effects of natural products on spore formation by *C. difficile* and other spore-forming pathogens^{235,236}.

Bacterial spores can remain viable in the environment for months to years and are difficult to eradicate as they are resistant to disinfectants commonly used in disinfection routines

^{222,224}. Spores can spread to patients via patient to patient contact and environmental contamination, potentially resulting in new and recurrent infections ^{224,237}. Therefore, the concurrent use of treatments preventing spore formation and conventional antimicrobials could reduce the possibility of transmission and the risk of recurrences. Moreover, some of these products may be suitable for use in the environment to reduce risk of infection by preventing spore formation.

4.3.2 Effect of products on vegetative outgrowth of *C. difficile*

An important factor in the initiation of CDI is spore germination, which is the first step after colonization ^{169,224,238}. The germination process is required in order to allow the outgrowth of vegetative cells ¹⁶⁹. Under suitable conditions, spore germination is induced, and the germinant receptor on the spore senses the presence of certain host-derived bile salt germinants such as taurocholic acid or cholic acid derivatives and amino acids (glycine and alanine) ²³⁹. In this study, none of the products inhibited spore germination, however, when the spore suspension was treated with artichoke extract, fresh onion bulb extract, *Leptospermum* honey, or allicin at certain concentrations, the outgrowth remained constant from 7 to 24 h, suggesting inhibition of the late stage of vegetative growth compared to controls. The mechanism of inhibitory activity of these products on the later stages of outgrowth is thus far unknown as little research has been conducted in this specific area.

Previously, the effect of antimicrobials such as fidaxomicin, vancomycin, oritavancin and metronidazole on *C. difficile* spore germination and outgrowth was assessed ^{170,240}. No effect on initiation of spore germination in the presence of supra-inhibitory concentrations of any of the antimicrobials was observed. However, spore outgrowth was inhibited to differing degrees when exposed to those antimicrobials, although the effect varied between strains ^{170,240}. Two previous studies have shown the inhibitory effect of several

muricholic acids and analogs of chenodeoxycholic acid on spore germination *in-vitro* ^{241,242}. The three muricholic acids, α -muricholic acid, β -muricholic acid and ω -muricholic acid, were suggested as potential inhibitors of *C. difficile* spore germination with likely impact on the growth of vegetative cells ²⁴¹. Although the natural products tested in the present study did not have any effect on spore germination, the impact on the outgrowth of vegetative cells in addition to an inhibitory effect on spore formation could be an important approach in the prevention of relapses of CDI. Moreover, preventing outgrowth would also inhibit toxin production and reduce or prevent other subsequent effects ¹⁷⁰.

4.3.3 Minor sporicidal activity of products against *C. difficile* spores

C. difficile spores serve as the infectious vehicle for transmission as well as a survival mechanism in the environment ²⁴³. Patients with CDI who have a prolonged hospital stay are likely to shed a large number of both vegetative cells and spores of *C. difficile* into their surroundings ²²⁶. Consequently, anything contaminated with spores including surfaces and hands of healthcare workers can become a vector of transmission ²³⁰.

Therefore, it is important to apply effective spore-killing treatments to reduce the rate of transmission. Among the five products assessed for sporicidal activity against *C. difficile*, peppermint oil (4% v/v) and *trans*-cinnamaldehyde (1% v/v) showed a reduction in spore count for the selected *C. difficile* strains after 24 h. However, these concentrations only resulted in $\log_{10}$ reduction values of ≤ 1 after 24 h; to achieve greater sporicidal activity higher concentrations of both products in addition to longer exposure times were required. Therefore, the findings from this assay may be of limited practical use. In a study by Lawrence and Palombo, 13 different essential oils were tested against *Bacillus subtilis* endospores and all showed some sporicidal activity ¹⁷¹. Of the 13 essential oils, cardamom, tea tree and juniper leaf oils showed the highest activity, reducing the number of viable spores by 3 logs at concentrations above 1% v/v. Several previous studies have

investigated the sporicidal effect of various disinfectants against *C. difficile* endospores²⁴⁴⁻²⁴⁶, but no published studies on the sporicidal activity of natural products against *C. difficile* spores were found. It has been suggested that some essential oil components, such as 1,8-cineole and α -terpinyl acetate are sporicidal by interfering with the spore membrane, weakening its structure and distorting the morphology of treated spores¹⁷¹. Endospores are inherently resistant to antimicrobials and there is currently no Environmental Protection Agency (EPA)-registered disinfectant available for inactivation of *C. difficile* spores. Ethanol-based disinfectants are commonly used for disinfecting contaminated surfaces in various settings, which strikingly have no effect on spore viability²²². The current recommendations for decontamination of spores are based on evidence available from the scientific literature²³⁰. Further studies are essential to enable the development of other alternatives containing novel disinfectant compounds. In this study, the products showing activity at concentrations that are safe for human consumption may be worthy of further investigation.

4.4 CONCLUSIONS

Overall, these results showed the inhibitory effect of several natural products on both sporulation and the vegetative outgrowth of *C. difficile in-vitro*. The products peppermint oil and *trans*-cinnamaldehyde showed minor sporicidal activity, which may not be practically relevant, as a long exposure time and high concentrations of these products were required to obtain a substantial reduction in *C. difficile* spore viability. However, the inhibition of sporulation as shown in this study may be a powerful way to reduce the transmission of CDI. In addition, inhibiting the conversion of spores to vegetative cells could be a contributing factor in reducing CDI recurrences.

Chapter 5

Effect of natural products on the production and activity of *C. difficile* toxins *in-vitro*

The following publication has arisen from this chapter:

Roshan N, Riley TV, Knight DR, Hammer KA. Effect of natural products on the production and activity of *Clostridium difficile* toxins *in-vitro*. *Scientific Reports* 2018; **8**:15735.

5.1 INTRODUCTION

CDI typically occurs following the disruption of normal enteric flora, post-antimicrobial exposure, leading to the proliferation and germination of *C. difficile* spores into vegetative cells with eventual toxin production in the intestine ²²⁴. The associated systemic inflammatory response is partly mediated by *C. difficile* toxins disseminating from the gastrointestinal tract ²⁴⁷. Toxins A (TcdA) and B (TcdB) are the main virulence factors responsible for diarrhoea and the physiological events associated with pseudomembranous colitis, thus the resulting infection strictly depends on toxins produced by *C. difficile* ^{33,44,248,249}. For many years, toxin A was thought to be the main trigger of disease pathogenesis, but now toxin B is proposed to be equally if not more important ²⁵⁰. A third toxin, binary toxin, has the ability to modify actin, however, there still remains much to be learned about its role in virulence ²⁵¹.

Toxins also induce apoptosis of gastrointestinal epithelial cells. *In-vitro* cellular apoptosis shows a clear, characteristic “cell rounding” phenotype or cytopathic effect (CPE) ^{31,252}. The changes in cell morphology caused by toxins are due to the disturbance of cell adhesion and cell-cell contacts, resulting in loss of integrity of intestinal epithelial cells and alteration of tight junction functions controlled by Rho proteins and actin ²⁵²⁻²⁵⁴. Very few studies have investigated the effect of naturally-occurring and plant-derived antimicrobials as possible anti-virulence strategies for reducing toxin production or activity. Toxins are the prime factor involved in the virulence of *C. difficile* and the pathogenesis of CDI ²⁵⁵, thus are considered major targets for developing therapeutic interventions.

5.1.1 Aims

Since CDI is a toxin-mediated disease, this chapter aimed to investigate the effects of natural products on *C. difficile* toxin production and activity *in-vitro*. The toxin protection

activity of natural products and their impact on toxin-mediated cytotoxicity was assessed using tissue culture and cytotoxicity assays. In addition, an ELISA assay was used to determine the effect of these products on toxin production by *C. difficile in-vitro*.

5.2 RESULTS

5.2.1 Toxin protection assay

Culture filtrates from all three toxigenic *C. difficile* strains (NCTC 13366, R11446 and ATCC 43598) showed CPE in Vero and HT-29 cells. However, only minor CPE was observed when the culture filtrate from *C. difficile* ATCC 43598 was applied to HT-29 cells. This was seen as cell rounding, membrane blebbing and loss of adhesion. A modal toxin titer that caused ~ 90% CPE was selected from three independent experiments for each toxigenic strains of *C. difficile* (Table 5.1). To avoid the gross effect of natural products on cells, the highest concentrations of each product showing no effect on cells were determined and are shown in Table 5.2. When culture filtrates and treatment were incubated together for 2 h prior to adding to the cells, only zingerone showed protection against any cytotoxic effect, determined visually (Figure 5.1) and confirmed by the neutral red uptake assay ($P \leq 0.007$) (Figure 5.3 and Supplementary Figures 1 and 2, Appendix II). A similar protective effect was observed when Vero cells were pre-incubated with zingerone for 2 h prior to the addition of culture filtrates. In contrast, immediate transfer of zingerone and culture filtrates to the wells containing Vero cells without prior incubation showed no protection against cytotoxicity. A similar protective effect against toxin activity was observed on HT-29 cell monolayers (Figure 5.2 and Supplementary Figures 1 and 2, Appendix II).

Table 5.1 *C. difficile* toxin titer required to achieve 90% CPE on Vero and HT-29 cells after 24 h incubation at 37°C with 5% CO₂.

<i>C. difficile</i> strain	Modal toxin titer (range)	
	Vero cells	HT-29 cells
NCTC 13366	1:524,288	1:262,144
	(1:524,288 – 1:2,097,152)	(1:131,072 - 1:524,288)
R11446	1:8,192	1:8,192
	(1:2,048 – 1:8,192)	(1:4,096 – 1:8,16,384)
ATCC 43598	1:512	1:8
	(1:512 – 1:1,024)	(1:8-1:32)

Table 5.2 Highest concentration of antimicrobial agents with no effect on Vero and HT-29 cells.

Antimicrobial agent	Units	Concentration	
		Vero cells	HT-29 cells
Fresh garlic bulb extract	% v/v	0.05	ND
Fresh ginger rhizome extract	% v/v	0.8	ND
Fresh onion bulb extract	% v/v	0.4	0.8
<i>Leptospermum</i> honey A	% w/v	0.5	0.5
<i>Leptospermum</i> honey B	% w/v	0.5	0.5
<i>Leptospermum</i> honey C	% w/v	0.5	0.5
Garlic clove powder	mg/ml	0.6	1.2
Ginger rhizome powder	mg/ml	18.8	ND
Cinnamon root powder	mg/ml	18.8	ND
Turmeric root powder	mg/ml	18.8	ND
Garlic tablet	mg/ml	2.3	ND
Ginger tablet	mg/ml	18.8	ND
Cinnamon tablet	mg/ml	18.8	ND
Turmeric tablet	mg/ml	18.8	ND
Artichoke capsule	mg/ml	2.3	ND
Aloe vera gel	% v/v	0.06	ND
Coconut oil	% v/v	0.13	ND
Peppermint oil	% v/v	0.03	ND
Allicin	mg/ml	1.2	ND
<i>trans</i> -Cinnamaldehyde	% v/v	0.01	0.004
Menthol	mg/ml	0.4	ND
Zingerone	mg/ml	1.2	1.2
Fidaxomicin	µg/ml	1	0.5

ND, not done.

Figure 5.1 Protection from cytopathic effect on Vero cells using microscopy (*C. difficile* culture filtrate and zingerone were incubated at 37°C for 2 h prior to being added to Vero cell monolayers). **1.** NCTC 13366; **2.** R11446; **3.** ATCC 43598; **A:** no culture filtrate; **B:** culture filtrate only; **C:** zingerone (1.2 mg/ml); **D:** zingerone (0.6 mg/ml); **E:** zingerone (0.3 mg/ml); Light microscopy $\times 40$; Scale: 50 μm .

Figure 5.2 Protection from cytopathic effect on HT-29 cells using microscopy (*C. difficile* culture filtrate and zingerone were incubated at 37°C for 2 h prior to being added to HT-29 cell monolayers). **1.** NCTC 13366; **2.** R11446; **A:** no culture filtrate; **B:** culture filtrate only; **C:** zingerone (1.2 mg/ml); **D:** zingerone (0.6 mg/ml); **E:** zingerone (0.3 mg/ml); Light microscopy $\times 40$; Scale: 50 μm .

Figure 5.3 Viability of (A) Vero cells and (B) HT-29 cells determined by the neutral red uptake assay (Culture filtrates and treatments were incubated for 2 h prior to being added to the cells).

Statistical significance: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to culture filtrate control.

5.2.2 Indirect effect of natural products on toxin-mediated cytotoxicity

The effect of all treatments on toxin-mediated cytotoxicity of *C. difficile* NCTC 13366 and R44116 on Vero cells was assessed (Table 5.3). The growth rate of bacteria treated with either $0.5 \times \text{MIC}$ or 0.25 MIC was compared to untreated controls by measuring OD_{600} to ensure that treatments did not significantly inhibit growth and that similar growth was achieved. Subsequently, those treatments showing a reduction in CPE were further tested against *C. difficile* ATCC 43598. Six out of 22 (27%) natural products showed a significant reduction in cytotoxicity on Vero cells compared to untreated culture filtrate from the three toxigenic *C. difficile* strains ($P \leq 0.047$) (Figure 5.4). Fresh onion bulb extract, garlic clove powder, *trans*-cinnamaldehyde and the three *Leptospermum* honeys at $0.5 \times$ and $0.25 \times \text{MICs}$ reduced the cytotoxicity titers for *C. difficile* filtrates by $\geq 70\%$ compared to the untreated controls after 48 h of incubation ($P \leq 0.001$). The garlic clove powder at $0.25 \times \text{MIC}$ did not show any reduction in CPE with HT-29 cells ($P < 0.05$).

5.2.3 Effect of products on *C. difficile* toxin production

The effects of natural products on toxin production were determined for those treatments showing an effect in either the direct or indirect toxin activity assays. Thus, the effect of seven products (fresh onion bulb extract, *trans*-cinnamaldehyde, the three *Leptospermum* honeys, garlic clove powder, and *trans*-cinnamaldehyde) on toxin production by the three toxigenic *C. difficile* strains was determined using ELISA. Toxin production for *C. difficile* strains in the presence of treatments relative to untreated cultures is shown in Figure 5.5. Five of the seven selected products, including fresh onion bulb extract, *trans*-cinnamaldehyde, and the three *Leptospermum* honeys, significantly reduced toxin production by all three toxigenic strains compared to the untreated controls ($P < 0.001$). Toxin production in the three toxigenic strains was inhibited by $\geq 40\%$ after 48 h of incubation.

Table 5.3 Modal cytotoxicity titer for *C. difficile* isolates inoculated with $0.5 \times \text{MIC}$ of antimicrobial agents.

Antimicrobial agents	Vero cells				HT-29 cells	
	Conc	<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 43598	<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446
Fresh garlic bulb extract	0.4% v/v	204800	1600	ND	ND	ND
Fresh ginger rhizome extract	50% v/v	409600	3200	ND	ND	ND
Fresh onion bulb extract	25% v/v	800***	200***	200*	800***	1600***
<i>Leptospermum</i> honey A	8% w/v	100***	200***	< 100***	800***	400***
<i>Leptospermum</i> honey B	8% w/v	100***	200***	< 100***	800***	400***
<i>Leptospermum</i> honey C	8% w/v	100***	200***	100***	800***	200***
Garlic clove powder	4.7 mg/ml	800***	400***	200*	51200***	1600***
Ginger rhizome powder	150 mg/ml	204800	1600	ND	ND	ND
Cinnamon root powder	37.5 mg/ml	204800	1600	ND	ND	ND
Turmeric root powder	150 mg/ml	409600	3200	ND	ND	ND
Garlic tablet	18.8 mg/ml	409600	3200	ND	ND	ND
Ginger tablet	150 mg/ml	409600	3200	ND	ND	ND
Cinnamon tablet	150 mg/ml	409600	1600	ND	ND	ND
Turmeric tablet	150 mg/ml	409600	3200	ND	ND	ND
Artichoke capsule	37.5 mg/ml	409600	3200	ND	ND	ND
Aloe vera gel	8% v/v	819200	3200	ND	ND	ND
Coconut oil	32% v/v	409600	3200	ND	ND	ND
Peppermint oil	2% v/v	409600	1600	ND	ND	ND
Allicin	1.2 mg/ml	409600	3200	ND	ND	ND
<i>trans</i> -Cinnamaldehyde	0.01% v/v	3200***	400**	200**	6400***	800***
Menthol	4.7 mg/ml	409600	1600	ND	ND	ND
Zingerone	4.7 mg/ml	409600	3200	800	51200***	6400***
Fidaxomicin	0.06 µg/ml	800***	200***	100***	1600***	400***
SDW		409600	3200	800	409600	6400
DMSO	2.5% v/v	409600	3200	800	409600	6400

ND, not done; Conc: concentration; Statistical significance: *<0.05, **<0.01, ***<0.001 compared to SDW.

Figure 5.4 Indirect effect of treatments on *C. difficile* cytotoxicity on (A) Vero cells and (B) HT-29 cells. Concentrations of agents used in the assay: Fresh onion bulb extract (25% and 12.5% v/v); Garlic clove powder (4.7 and 2.3 mg/ml); *Leptospermum* honey (A), (B) and (C) (8% and 4% v/v); *trans*-Cinnamaldehyde (0.01% and 0.005% v/v); Zingerone (4.7 and 2.3 mg/ml); Fidaxomicin (0.06 µg/ml). Statistical significance: Δ = $P < 0.05$, O = $P < 0.01$, * = $P < 0.001$ compared to controls. *LH, *Leptospermum* honey; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

Figure 5.5 Effect of treatments on *C. difficile* toxin production. Concentrations of agents used in the assay: Fresh onion bulb extract (25% and 12.5% v/v); Garlic clove powder (4.7 and 2.3 mg/ml); *Leptospermum* honey (A), (B) and (C) (8% and 4% v/v); *trans*-Cinnamaldehyde (0.01% and 0.005% v/v); Zingerone (4.7 and 2.3 mg/ml); Fidaxomicin (0.06 µg/ml). Statistical significance: Δ = $P < 0.05$, O = $P < 0.01$, * = $P < 0.001$ compared to controls.

*LH, *Leptospermum* honey; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

5.3 DISCUSSION

In general, toxins are considered key targets in the search of new treatments fighting infections, as they are important in the establishment of infection by triggering numerous cellular processes in the host ^{31,247}. Toxins are the major virulence factors of *C. difficile* and are the main initiators of gastrointestinal disease, causing inflammation and mucosal damage in the intestine ²⁸. Conventional antimicrobials are the drugs of choice for treating bacterial infection, however, there are disadvantages to using antimicrobials including the development of antimicrobial resistance and the disruption of normal microbiota ^{256,257}. Naturally occurring antimicrobials may represent a viable approach for developing anti-virulence agents, and specifically targeting toxin production. Hence, it is worthwhile to investigate alternative toxin-active therapeutic agents to counteract the toxin-mediated virulence of *C. difficile* ¹⁷².

5.3.1 Protection against cytotoxicity effect

5.3.1.1 Direct activity

Historically, the C-terminal domain of TcdA has been recognized as a receptor-binding structure due to its homology to the carbohydrate binding region of streptococcal glycosyltransferases ²⁵⁸. This region consists of combined repetitive oligopeptides (CROPs), comprising receptor-binding domain of TcdA and antibodies that recognise this region could inhibit TcdA-induced haemagglutination ^{258,259}. Later, it was proposed that the region N-terminally adjacent to the CROPs also contributes to receptor binding ²⁵⁹. In addition, the lack of CROPs in TpeL as a large clostridial glucosyltransferase from *C. perfringens* further questioned whether the CROPs are dispensable for the function of TcdA and TcdB ²⁵⁸. In 2003, the generation of recombinant deletion mutants of TcdA and TcdB without CROPs showed a cytopathic effect in cell culture assays, further

suggesting multiple and complex receptor-binding domains involved in clostridial glucosylating toxin uptake ^{259,260}.

In this study, assessing the direct protective effect of products on *C. difficile* toxin activity showed similar findings with both cell lines. The ginger component zingerone protected cells against the effects of *C. difficile* toxin. Protection against toxin activity was evident when culture filtrates and zingerone were incubated together for 2 h prior to being added to the cells, in addition to when the cells were pre-incubated with zingerone prior to addition of culture filtrates. However, the simultaneous addition of culture filtrates and zingerone to wells containing either Vero or HT-29 cells showed no significant protection. Therefore, it is possible that zingerone may provide protection against CPE by blocking either the toxin-binding sites on toxin molecules or the host cell receptors.

An earlier study by Chen *et al.* investigated the effect of ginger extract and its bioactive components on inhibition of enterotoxigenic *E. coli* heat-labile enterotoxin (LT)-induced diarrhoea in mice ¹⁵⁶. Ginger extract blocked the binding of LT to the G_{M1} cell-surface receptor, resulting in inhibition of fluid accumulation in the ileal loops of mice. Based on a further investigation of the biologically-active constituents of ginger, it was concluded that zingerone was the active component responsible for the antidiarrhoeal effect of ginger ¹⁵⁶. Another study has reported the inhibitory effect of zingerone on colonic motility in rats, directly affecting the smooth muscles ²⁶¹. Considering the capacity for zingerone to reduce fluid secretion, it has been suggested as a potential compound for treating diarrhoea and other gastrointestinal disorders.

Allicin, as an active component of garlic has been previously shown to inhibit the haemolytic activity of pneumolysin, a toxin produced by *Streptococcus pneumoniae* in a concentration- and time-dependent manner ²⁶². This showed that pneumolysin could be a novel target for allicin and provided a new line of investigation for pneumococcal disease ²⁶². Anti-toxin therapy against *C. difficile* has been tried with antibodies, but not

natural products^{86,263,264} and monoclonal antibodies have been approved as an option to reduce reoccurrences²⁶⁵. Bezlotoxumab is a human monoclonal antibody that acts directly against *C. difficile* toxin B, preventing intestinal epithelial damage. It has emerged as an adjunctive therapy for preventing recurrent CDI^{263,264}. Thus, investigating other alternative therapies such as natural products that target prime virulence factors involved in CDI pathogenesis will be valuable in reducing the burden of CDI.

5.3.1.2 Indirect activity

Results from the indirect toxin-mediated cytotoxicity assay and ELISAs showed an inhibitory effect for several products (fresh onion bulb extract, three *Leptospermum* honeys, and *trans*-cinnamaldehyde at $0.5 \times$ and $0.25 \times$ MICs), on both toxin production and activity. Garlic clove powder at 0.5 and $0.25 \times$ MIC reduced toxin activity on Vero cells, but only at $0.5 \times$ MIC with HT-29 cells. Furthermore, results obtained by ELISA did not indicate any significant decrease in toxin production with garlic powder compared to the untreated controls.

The effect of plant extracts at either sub-lethal or sub-inhibitory concentrations against virulence factors of Gram-positive bacteria has been previously reported²⁶⁶⁻²⁶⁹. *trans*-Cinnamaldehyde (0.38 mM; 0.05 mg/ml) reduced both *C. difficile* toxin production and activity *in-vitro*²⁶⁷. Another study by Upadhyay *et al.* reported the effect of *trans*-cinnamaldehyde (0.5 mM) on down-regulating the *hly* and *prfA* genes that code for toxin production and a transcriptional regulator in *Listeria monocytogenes*²⁶⁸. As reported by Qiu *et al.* capsaicin, the main amide-derived alkaloid in chili decreases the production of α -toxin by methicillin resistant *S. aureus* (MRSA) at sub-inhibitory concentrations²⁶⁹. Additionally, *in-vivo* studies showed that capsaicin protected mice from MRSA pneumonia²⁶⁹.

Moreover, several antimicrobials such as fidaxomicin, its major metabolite OP-1118, metronidazole and vancomycin have been investigated for activity against *C. difficile* toxins²⁷⁰. Both fidaxomicin and OP-1118 at $0.25 \times \text{MIC}$ inhibited toxin production by $\geq 60\%$ following one week of culture. In addition, the expression of toxin genes was almost completely inhibited after exposure to either fidaxomicin ($2 \times \text{MIC}$) or OP-1118 ($2.5 \times \text{MIC}$). However, neither metronidazole nor vancomycin had an inhibitory effect on *C. difficile* toxin production or gene expression²⁷⁰. In the current study, fidaxomicin was used as a positive control and a reduction in both *C. difficile* toxin production and activity was observed after treatment with this antimicrobial.

5.3.2 Activity of zingerone

Although zingerone showed a direct protective effect against *C. difficile* toxins, it did not have any effect on toxin-mediated cytotoxicity or in toxin production assays. One possible explanation is that hypothetically, zingerone could have a reasonably rapid bactericidal activity which may degrade over time, leading to a loss of activity. However, there is no published literature to support this hypothesis. During both indirect toxin-mediated cytotoxicity and toxin production assays, there was no direct exposure between toxin and the treatments and it could have taken some time for *C. difficile* to initiate toxin production. Thus, by the time toxins were produced, the activity of zingerone may have been minimal.

5.4 CONCLUSIONS

The *in-vitro* activity of a number of food-grade and plant-derived products against *C. difficile* toxins is highlighted in this chapter. This suggests that several natural products may have potential as either alternative or complementary treatment options for CDI. Application of various anti-virulence strategies such as the disruption of toxin production

and toxin-mediated pathology in patients with CDI may be a possible approach in either controlling or treating infection, or reducing recurrences.

Chapter 6

Mechanisms of action of natural products against *C. difficile*

The following publication has arisen from this chapter:

Roshan N, Riley TV, Knight DR, Steer J, Hammer KA. Natural products show diverse mechanisms of action. *Journal of Applied Microbiology* 2018; **126**: 468-79.

6.1 INTRODUCTION

Treatment strategies involving the use of natural compounds derived from plant extracts are gaining importance and this is particularly relevant given that the current options for *C. difficile* treatment are limited²⁷¹. Anti-*C. difficile* agents with novel modes of action, enhanced specificity, high potency and better cost-benefit will be superior over metronidazole, vancomycin, and fidaxomicin in both treating and preventing primary and recurrent CDI⁶⁹. Many natural products have great antimicrobial potential against drug-resistant pathogens and, unlike the current antimicrobials, multi-component natural products are less likely to enhance the emergence of AMR^{194,271}.

Understanding the mechanisms of action of plant extracts, especially their biocidal properties, can help with their medical and environmental application²⁷². In general, conventional antimicrobials exert their effects on microbes by influencing the synthesis of the cell wall, proteins and nucleic acids, or metabolite activity²⁷³. It would be a great advantage if the mode of action of alternative or complementary antimicrobial agents was distinct from the known mechanisms of action of conventional antimicrobials²⁷⁴.

6.1.1 Aims

The purpose of this chapter was to investigate the mechanisms of action of nine compounds, namely cinnamon root powder, peppermint oil, *trans*-cinnamaldehyde, menthol, zingerone, allicin, fresh garlic bulb extract, garlic clove powder and *Leptospermum* honey (MGO 514+) that were previously shown to have activity against *C. difficile in-vitro*. Several possible mechanisms or modes of action were investigated, including examining time-kill kinetics for the selected natural compounds as well as their effect on ATP biosynthesis. The effect of these products on the membrane integrity of *C. difficile* was also assessed. In addition, the inhibitory effect of selected compounds on prokaryotic translation/protein synthesis was investigated. Lastly, the possibility of

antimicrobial cross-resistance was examined by determining the inhibitory effects of natural products against a panel of *C. difficile* strains previously characterized for AMR phenotype and associated genotype.

6.2 RESULTS

6.2.1 Time-kill assay

Time-kill assays were conducted against both logarithmic- and stationary-phase cultures of *C. difficile* strains ATCC 700057 and NCTC 13366, with various concentrations of treatments. Previous results demonstrated that five of the 22 products showed bactericidal activity against stationary-phase *C. difficile*. These were cinnamon root powder, peppermint oil, *trans*-cinnamaldehyde, menthol and zingerone, and these products were therefore selected for testing in time-kill assays. There were substantial reductions in the viability of both *C. difficile* strains following 24 h exposure to these products (Tables 6.1 and 6.2). A $\geq 3 \log_{10}$ reduction in bacterial count was considered to be biologically significant killing activity. Treatment with peppermint oil at $\geq 1 \times \text{MIC}$ resulted in a $\log_{10}$ reduction of ≥ 3 against both log- and stationary-phase *C. difficile* after 24 h. Similarly, *trans*-cinnamaldehyde at nearly all concentrations showed a comparable pattern of killing, except at $1 \times \text{MIC}$ against stationary-phase *C. difficile* strain NCTC 13366 where there was a $\log_{10}$ reduction of 2.9 after 24 h. Treatment with both zingerone and menthol resulted in $\log_{10}$ reductions of ≥ 3 at $\geq 2 \times \text{MIC}$ and $\geq 4 \times \text{MIC}$, respectively, after 24 h incubation with either log- or stationary-phase *C. difficile*. Treatment with cinnamon root powder at $\geq 4 \times \text{MIC}$ ($\geq 75 \text{ mg/ml}$) resulted in a $\log_{10}$ reduction of ≥ 3.2 after 24 h exposure for both log-phase *C. difficile* strains. Also, a $\log_{10}$ reduction of ≥ 3.5 occurred after treatment with cinnamon root powder at $\geq 2 \times \text{MIC}$ ($\geq 75 \text{ mg/ml}$) against the stationary-phase *C. difficile* after 24 h.

Table 6.1 Mean log₁₀ CFU/ml reductions for *C. difficile* NCTC 13366 following of exposure to products.

Treatment	Concentration	<i>Clostridium difficile</i> NCTC 13366									
		1h		2h		4h		6h		24h	
		EXP	STA	EXP	STA	EXP	STA	EXP	STA	EXP	STA
Cinnamon root powder	1 × MIC	0.6 ± 0.3	1.5 ± 0.4	1.2 ± 0.2	1.6 ± 0.4	1.2 ± 0.3	2.1 ± 0.2	1.3 ± 0.2	2.4 ± 0.3	1.6 ± 0.3	2.6 ± 0.2
	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	1 × MIC	1.9 ± 0.1	1.4 ± 0.4	2.2 ± 0.2	1.7 ± 0.3	2.6 ± 0.3	2.2 ± 0.4	3.0 ± 0.1	2.4 ± 0.3	3.4 ± 0.1	3.4 ± 0.5
	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
<i>trans</i> -Cinnamaldehyde	1 × MIC	2.4 ± 0.8	0.7 ± 0.4	2.3 ± 0.9	1.8 ± 0.3	2.9 ± 0.5	2.0 ± 0.4	3.0 ± 0.5	2.6 ± 0.2	3.0 ± 0.6	2.9 ± 0.2
	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	1 × MIC	0.5 ± 0.3	1.3 ± 0.8	1.2 ± 0.3	2.0 ± 0.2	1.5 ± 0.4	2.4 ± 0.2	2.4 ± 1.0	2.4 ± 0.2	2.9 ± 0.8	3.5 ± 0.4
	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	1 × MIC	0.9 ± 0.2	0.6 ± 0.3	1.6 ± 0.4	0.9 ± 0.1	2.1 ± 0.8	1.1 ± 0.6	1.8 ± 0.5	1.3 ± 0.4	-0.1 ± 0.7	-0.1 ± 0.2
	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
SDW	-	-0.1 ± 0.1	-0.03 ± 0.1	-0.2 ± 0.1	-0.2 ± 0.3	-0.4 ± 0.1	-0.3 ± 0.3	-0.8 ± 0.3	-0.6 ± 0.3	-1.5 ± 0.2	-1.6 ± 0.5
DMSO	2.5% v/v	-0.3 ± 0.3	-0.1 ± 0.2	-0.43 ± 0.4	-0.4 ± 0.3	-0.7 ± 0.4	-0.7 ± 0.3	-0.9 ± 0.5	-0.9 ± 0.3	-1.9 ± 0.3	-1.7 ± 0.1

EXP, exponential; STA, stationary; MIC, minimum inhibitory concentration; NA, not applicable; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

A ≥ 3 log₁₀ reduction is highlighted in bold.

Table 6.2 Mean log₁₀ CFU/ml reductions for *C. difficile* ATCC 700057 following of exposure to products.

Treatment	Concentration	<i>Clostridium difficile</i> ATCC 700057									
		1h		2h		4h		6h		24h	
		EXP	STA	EXP	STA	EXP	STA	EXP	STA	EXP	STA
Cinnamon root powder	1 × MIC	0.9 ± 0.1	0.5 ± 0.2	2.3 ± 0.4	1.4 ± 0.4	2.3 ± 0.5	1.8 ± 0.9	2.3 ± 0.5	2.4 ± 0.7	2.4 ± 0.4	2.7 ± 0.7
	2 × MIC	1.4 ± 0.6	1.7 ± 0.5	1.6 ± 0.9	2.3 ± 0.4	2.3 ± 0.6	3.1 ± 0.3	2.5 ± 0.6	3.3 ± 0.4	2.6 ± 0.7	3.5 ± 0.2
	4 × MIC	1.7 ± 0.9	NA	2.6 ± 0.3	NA	2.8 ± 0.2	NA	2.9 ± 0.2	NA	3.2 ± 0.2	NA
	8 × MIC	2.3 ± 0.4	NA	2.8 ± 0.5	NA	3.3 ± 0.6	NA	3.4 ± 0.8	NA	4.3 ± 0.4	NA
Peppermint oil	1 × MIC	2.0 ± 0.3	1.4 ± 0.4	2.7 ± 0.5	1.5 ± 0.3	3.0 ± 0.4	2.5 ± 0.3	3.2 ± 0.2	2.7 ± 0.2	3.5 ± 0.1	3.6 ± 0.5
	2 × MIC	2.1 ± 0.3	1.9 ± 0.5	2.9 ± 0.7	2.0 ± 0.6	3.2 ± 0.4	2.8 ± 0.2	3.4 ± 0.3	2.9 ± 0.2	3.7 ± 0	3.6 ± 0.5
	4 × MIC	2.5 ± 0.5	2.3 ± 0.5	3.0 ± 0.9	2.3 ± 0.6	3.8 ± 0.2	3.0 ± 0.4	3.9 ± 0.1	3.1 ± 0.5	4.0 ± 0	3.8 ± 0.2
	8 × MIC	2.7 ± 0.4	NA	3.1 ± 0.8	NA	3.8 ± 0.2	NA	4.1 ± 0.1	NA	4.2 ± 0.2	NA
<i>trans</i> -Cinnamaldehyde	1 × MIC	1.0 ± 0.7	0.6 ± 0.4	1.7 ± 0.6	1.9 ± 0.2	2.9 ± 1.2	2.2 ± 0.5	3.2 ± 1.2	2.3 ± 0.4	3.4 ± 1.2	3.2 ± 0.7
	2 × MIC	1.5 ± 0.7	0.7 ± 0.3	2.6 ± 0.8	2.0 ± 0.1	3.9 ± 0.5/	2.6 ± 0.4	4.2 ± 0.2	2.6 ± 0.4	4.3 ± 0	3.3 ± 0.4
	4 × MIC	2.6 ± 0.2	1.3 ± 0.6	3.9 ± 0.3	2.4 ± 0.4	4.2 ± 0.2/	3.0 ± 0.0	4.3 ± 0.0	3.1 ± 0.1	4.3 ± 0	3.5 ± 0.6
	8 × MIC	3.3 ± 0.9	1.8 ± 0.2	4.2 ± 0.2	2.5 ± 0.4	4.2 ± 0.1	3.0 ± 0.0	4.3 ± 0.0	3.1 ± 0.1	4.3 ± 0	3.7 ± 0.7
Menthol	1 × MIC	0.1 ± 0.1	1.3 ± 0.3	0.4 ± 0.3	1.9 ± 0.4	1.9 ± 1.0	2.1 ± 0.2	2.2 ± 0.8	2.3 ± 0.3	2.7 ± 0.7	2.7 ± 0.6
	2 × MIC	0.5 ± 0.4	1.8 ± 0.2	1.0 ± 0.7	2.1 ± 0.2	1.9 ± 0.9	2.4 ± 0.3	2.0 ± 0.4	2.8 ± 0.2	2.5 ± 0.6	3.5 ± 0.3
	4 × MIC	0.8 ± 0.7	2.2 ± 0.2	3.1 ± 0.8	2.4 ± 0.1	3.7 ± 0.4	2.7 ± 0.2	3.6 ± 0.5	3.1 ± 0.2	3.7 ± 0.4	3.7 ± 0.4
	8 × MIC	2.9 ± 0.6	2.6 ± 0.2	3.8 ± 0.4	2.6 ± 0.2	3.7 ± 0.6	3.1 ± 0.2	4.1 ± 0.2	3.1 ± 0.2	4.1 ± 0.1	3.7 ± 0.4
Zingerone	1 × MIC	0.0 ± 0.0	0.3 ± 0.3	0.7 ± 0.3	0.5 ± 0.3	0.5 ± 0.4	0.7 ± 0.3	0.5 ± 0.4	1.2 ± 0.3	-0.3 ± 0.5	-0.02 ± 0.2
	2 × MIC	1.3 ± 0.6	1.8 ± 0.2	1.6 ± 0.5	2.2 ± 0.3	2.3 ± 0.8	2.5 ± 0.2	2.9 ± 1.0	3.2 ± 0.2	3.2 ± 1.1	3.2 ± 0.2
	4 × MIC	2.1 ± 1.0	2.4 ± 0.4	2.0 ± 1.0	2.7 ± 0.4	3.2 ± 0.7	2.8 ± 0.4	3.5 ± 0.8	3.2 ± 0.2	3.6 ± 0.9	3.3 ± 0.4
	8 × MIC	3.0 ± 0.0	2.4 ± 0.3	3.3 ± 0.6	2.7 ± 0.4	3.9 ± 0.5	3.1 ± 0.3	4.1 ± 0.3	3.3 ± 0.1	3.9 ± 0.8	3.3 ± 0.4
SDW	-	-0.1 ± 0.1	-0.1 ± 0.1	-0.4 ± 0.4	-0.6 ± 0.3	-0.6 ± 0.5	-0.9 ± 0.6	-0.8 ± 0.5	-1.3 ± 0.6	-1.7 ± 0.2	-1.6 ± 0.3
DMSO	2.5% v/v	-0.4 ± 0.4	-0.2 ± 0.3	-0.5 ± 0.4	-0.4 ± 0.3	-0.6 ± 0.4	-0.7 ± 0.4	-1.2 ± 0.2	-1.4 ± 0.3	-1.7 ± 0.1	-1.7 ± 0.1

EXP, exponential; STA, stationary; MIC, minimum inhibitory concentration; NA, not applicable; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

A ≥ 3 log₁₀ reduction is highlighted in bold.

6.2.2 ATP-leakage assay

Figures 6.1A and 6.1B show the effect of different concentrations of each of the five bactericidal products on leakage of ATP from logarithmic-phase cultures of *C. difficile* NCTC 13366. Treatment with the positive control nisin (4 µg/ml) resulted in significant changes in levels of both intracellular and extracellular ATP ($P < 0.001$) compared to the untreated controls (SDW and DMSO) only after 1 h of incubation. In contrast, treatment with the negative control vancomycin (8 µg/ml) did not result in any ATP loss ($P = 0.118$). Treatment with each of the five products at most concentrations resulted in a significant decrease in intracellular ATP only after 1 h of treatment ($P \leq 0.013$). The only exceptions were cinnamon root powder and zingerone at $1 \times \text{MIC}$, which did not show any significant decrease in intracellular ATP even after 6 h of exposure ($P \geq 0.279$). Treatment with menthol and peppermint oil at both $4 \times \text{MIC}$ and $8 \times \text{MIC}$ resulted in a significant increase in levels of extracellular ATP after 1 h of exposure ($P < 0.04$). Most of products at concentrations of $\leq 2 \times \text{MIC}$ did not show any significant increase in extracellular ATP up to 2 h ($P \geq 0.06$).

6.2.3 Bacteriolysis

Bacteriolysis was quantified by determining the optical density (620nm) of treated and untreated cultures of *C. difficile* strains ATCC 700057 and NCTC 13366 and expressing this as a proportion of the time zero optical density ($\% t_n/t_0$) (Table 6.3). Cell lysis was not observed with any of the five bactericidal products at concentrations well above the MBC. No significant lysis occurred for the untreated controls (SDW and DMSO) or the negative control (vancomycin; 8 µg/ml). Only the positive control, which was treated with Triton X-100 (10% v/v), showed a significant reduction in the OD₆₂₀ to 64.9% after 6 h when compared to the untreated control (SDW; ~ 85%) ($P \leq 0.023$).

Figure 6.1A Effect (mean $\pm$ SD) of five bactericidal products and comparators on the leakage of ATP from *C. difficile* NCTC 13366. Concentration of treatments used in this assay: Nisin $8 \times$ MIC ($4 \mu\text{g/ml}$), vancomycin $8 \times$ MIC ($8 \mu\text{g/ml}$), cinnamon root powder $8 \times$ MIC (150 mg/ml), peppermint oil $8 \times$ MIC ($16\% \text{ v/v}$), *trans*-cinnamaldehyde $8 \times$ MIC ($1.6\% \text{ v/v}$), menthol $8 \times$ MIC (18.8 mg/ml), zingerone $8 \times$ MIC (37.5 mg/ml).

Figure 6.1B Effect (mean $\pm$ SD) of five bactericidal products and comparators on the leakage of ATP from *C. difficile* NCTC 13366. Concentration of treatments used in this assay: [A] Cinnamon root powder 4 $\times$ MIC (75 mg/ml), peppermint oil 4 $\times$ MIC (8% v/v), *trans*-cinnamaldehyde 4 $\times$ MIC (0.08% v/v), menthol 4 $\times$ MIC (9.4 mg/ml), zingerone 4 $\times$ MIC (18.8 mg/ml). [B] Cinnamon root powder 2 $\times$ MIC (37.5 mg/ml), peppermint oil 2 $\times$ MIC (4% v/v), *trans*-cinnamaldehyde 2 $\times$ MIC (0.04% v/v), menthol 2 $\times$ MIC (4.7 mg/ml), zingerone 2 $\times$ MIC (9.4 mg/ml); [C] Cinnamon root powder 1 $\times$ MIC (18.8 mg/ml), peppermint oil 1 $\times$ MIC (2% v/v), *trans*-cinnamaldehyde 1 $\times$ MIC (0.02% v/v), menthol 1 $\times$ MIC (2.3 mg/ml), zingerone 1 $\times$ MIC (4.7 mg/ml).

Table 6.3 Bacteriolytic activity of natural products against *C. difficile* expressed as percentage relative to time zero.

Treatment	Conc.	<i>Clostridium difficile</i> NCTC 13366			<i>Clostridium difficile</i> ATCC 700057		
		1h	2h	6h	1h	2h	6h
Cinnamon root powder	75 mg/ml	99.7 ± 5.4	101.9 ± 0.9	101.3 ± 4.2	98.6 ± 2.3	95.7 ± 4.9	101.2 ± 7.3
Peppermint oil	32% v/v	101.3 ± 1.2	101.1 ± 0.9	103.5 ± 4.1	101.5 ± 0.9	100.4 ± 2.0	99.3 ± 9.4
<i>trans</i> -Cinnamaldehyde	1.6% v/v	97.9 ± 13.6	101.1 ± 6.2	98.5 ± 3.7	100.1 ± 0.5	101.1 ± 0.9	99.6 ± 12.6
Menthol	75 mg/ml	95.5 ± 5.3	98.3 ± 2.8	90.2 ± 9.1	84.4 ± 9.1	87.1 ± 9.9	99.2 ± 11.1
Zingerone	75 mg/ml	102.1 ± 7.1	96.6 ± 3.1	100.5 ± 16.9	98.7 ± 1.9	99.7 ± 1.6	99.8 ± 8.5
Triton X-100	10% v/v	94.9 ± 4.5	75.4 ± 8.4	64.9 ± 1.6	95.0 ± 1.4	68.2 ± 7.3	64.9 ± 6.5
Vancomycin	8 µg/ml	91.0 ± 12.9	97.8 ± 0.5	87.3 ± 0.9	88.3 ± 11.7	87.2 ± 10.4	81.5 ± 5.3
DMSO	2.5% v/v	98.9 ± 1.3	96.3 ± 1.6	89.5 ± 4.2	100.5 ± 2.1	91.6 ± 7.1	82.8 ± 1.7
SDW	-	97.6 ± 5.1	93.8 ± 3.5	85.6 ± 3.1	98.7 ± 0.5	94.5 ± 3.7	85.0 ± 7.2

SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

Values in bold differ significantly from controls (SDW and DMSO), $P < 0.05$.

6.2.4 Permeability assays

Results of the three different cell permeability assays are summarized in Tables 6.4 and 6.5. Stationary-phase cultures of the two strains *C. difficile* ATCC 700057 and NCTC 13366 were exposed to various concentrations of each of the five bactericidal products.

6.2.4.1 Loss of 260nm-absorbing material.

The amount of intracellular material released from cells into the suspension was determined by measuring the optical density of the cell-free supernatant at 260nm. None of the five products caused any significant increase in OD₂₆₀ after 1 h of exposure, in comparison to the untreated controls (SDW and DMSO). However, treatment with the positive control nisin (4 µg/ml) resulted in a significant increase in OD₂₆₀ after only 1 h for *C. difficile* NCTC 13366 ($P < 0.03$). After a longer incubation time (6 h), most products at concentrations of $\geq 2 \times \text{MIC}$ resulted in significant increases in OD₂₆₀ for both strains (*C. difficile* ATCC 700057 and NCTC 13366) ($P < 0.05$). However, no significant increase in OD₂₆₀ was observed after treatment with cinnamon root powder ($P > 0.05$) or the negative control substance vancomycin (8 µg/ml).

6.2.4.2 Protein leakage assay

The Bradford assay was used to determine the amount of protein released from the treated cells. Treatment with peppermint oil, *trans*-cinnamaldehyde, menthol, and zingerone resulted in a significant increase in extracellular protein after 1 h of exposure compared to the untreated controls (SDW and DMSO) ($P \leq 0.04$). Additionally, a significant increase in extracellular protein was observed after 6 h exposure to cinnamon root powder at $2 \times \text{MIC}$ (150 mg/ml) ($P < 0.01$) for both *C. difficile* strains. Treatment with the positive control nisin (4 µg/ml) resulted in a significant increase in extracellular protein for both strains after 2 h exposure ($P < 0.04$), while treatment with the negative control

Table 6.4 Effect of products on membrane integrity of *C. difficile* NCTC 13366, measured by an increase in extracellular 260nm absorbing materials, protein leakage and propidium iodide uptake assays.

Treatment	Extracellular 260nm absorbing material (OD ₂₆₀)				Protein leakage assay (extracellular leakage mg/ml)				Propidium iodide uptake (RFUs)		
	0h	1h	2h	6h	0h	1h	2h	6h	1h	2h	6h
Cinnamon root powder											
1 × MIC (75 mg/ml)	0.00 ± 0.00	0.03 ± 0.02	0.06 ± 0.03	0.07 ± 0.02	0.0 ± 0.0	7.0 ± 2.3	3.0 ± 1.8	24.8 ± 5.7	324 ± 53	376 ± 136	602 ± 128
2 × MIC (150 mg/ml)	0.00 ± 0.00	0.03 ± 0.02	0.07 ± 0.02	0.11 ± 0.01	0.0 ± 0.0	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)	0.00 ± 0.00	0.01 ± 0.00	0.04 ± 0.02	0.11 ± 0.01	0.0 ± 0.0	19.2 ± 6.8	23.6 ± 5.2	74.3 ± 3.9	241 ± 60	444 ± 108	1206 ± 186
2 × MIC (16% v/v)	0.00 ± 0.00	0.02 ± 0.01	0.05 ± 0.01	0.14 ± 0.02	0.0 ± 0.0	29.6 ± 9.1	62.9 ± 2.7	90.7 ± 11.2	431 ± 156	548 ± 111	1387 ± 294
4 × MIC (32% v/v)	-0.00 ± 0.00	0.03 ± 0.01	0.08 ± 0.02	0.24 ± 0.08	0.0 ± 0.0	32.8 ± 11.0	74.1 ± 2.6	99.5 ± 8.7	256 ± 14	399 ± 142	1061 ± 228
<i>trans</i> -Cinnamaldehyde											
1 × MIC (0.02% v/v)	0.01 ± 0.01	0.01 ± 0.00	0.04 ± 0.02	0.07 ± 0.01	0.0 ± 0.0	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)	0.00 ± 0.00	0.02 ± 0.02	0.06 ± 0.02	0.12 ± 0.02	0.0 ± 0.0	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)	0.00 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.15 ± 0.01	0.0 ± 0.0	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)	0.00 ± 0.00	0.02 ± 0.01	0.05 ± 0.00	0.17 ± 0.07	0.0 ± 0.0	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)	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.05 ± 0.00	0.0 ± 0.0	0.0 ± 0.0	36.0 ± 6.9	42.2 ± 17.6	308 ± 12	315 ± 99	223 ± 21
2 × MIC (18.8 mg/ml)	0.00 ± 0.00	0.02 ± 0.01	0.04 ± 0.01	0.11 ± 0.01	0.0 ± 0.0	24.6 ± 14.8	37.2 ± 12.6	51.1 ± 13.3	287 ± 21	287 ± 88	487 ± 99
4 × MIC (37.5 mg/ml)	0.00 ± 0.00	0.02 ± 0.01	0.04 ± 0.02	0.12 ± 0.02	0.0 ± 0.0	37.7 ± 4.8	51.5 ± 16.7	62.3 ± 22.6	337 ± 8.9	463 ± 101	513 ± 88
8 × MIC (75 mg/ml)	0.00 ± 0.00	0.04 ± 0.03	0.06 ± 0.02	0.14 ± 0.03	0.0 ± 0.0	24.2 ± 8.9	48.8 ± 20.1	76.6 ± 13.5	461 ± 84	499 ± 88	987 ± 44

Table 6.4 (Cont.) Effect of products on membrane integrity of *C. difficile* NCTC 13366, measured by an increase in extracellular 260nm absorbing materials, protein leakage, and propidium iodide uptake assays.

Treatment	Extracellular 260nm absorbing material (OD ₂₆₀)				Protein leakage assay (protein leakage mg/ml)				Propidium iodide uptake (RFUs)		
	0h	1h	2h	6h	0h	1h	2h	6h	1h	2h	6h
Zingerone											
1 × MIC (9.4 mg/ml)	0.00 ± 0.01	0.02 ± 0.00	0.05 ± 0.02	0.08 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	698 ± 73	595 ± 20	990 ± 99
2 × MIC (18.8 mg/ml)	0.00 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.12 ± 0.01	0.0 ± 0.0	11.5 ± 5.4	5.2 ± 3.3	3.1 ± 3.3	739 ± 67	574 ± 19	1247 ± 29
4 × MIC (37.5 mg/ml)	0.00 ± 0.00	0.04 ± 0.02	0.06 ± 0.01	0.16 ± 0.07	0.0 ± 0.0	5.7 ± 3.3	20.3 ± 9.6	35.2 ± 15.3	1263 ± 425	919 ± 21	1301 ± 12
8 × MIC (75 mg/ml)	0.00 ± 0.01	0.03 ± 0.01	0.06 ± 0.02	0.16 ± 0.03	0.0 ± 0.0	38.9 ± 13.9	50.7 ± 13.4	41.7 ± 15.1	1101 ± 418	1601 ± 122	1214 ± 57
Nisin (4 µg/ml)	0.00 ± 0.01	0.06 ± 0.02	0.10 ± 0.00	0.16 ± 0.04	0.0 ± 0.0	6.7 ± 4.1	29.8 ± 5.6	53.7 ± 11.4	1448 ± 59	1254 ± 35	3611 ± 284
Vancomycin (8 µg/ml)	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.01	0.0 ± 0.0	4.1 ± 2.4	16.7 ± 4.7	18.5 ± 7.2	181 ± 16	175 ± 54	218 ± 88
SDW	-0.00 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	1.6 ± 2.9	147 ± 26	185 ± 45	206 ± 65
DMSO (2.5% v/v)	0.00 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 2.1	134 ± 37	185 ± 45	166 ± 53

SDW, sterile distilled water; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; RFUs: relative fluorescence units. Values in bold differ significantly from controls (SDW and DMSO), $P < 0.05$.

Table 6.5. Effect of products on membrane integrity of *C. difficile* ATCC 700057, measured by an increase in extracellular 260nm absorbing materials, protein leakage and propidium iodide uptake assays.

Treatment	Extracellular 260nm absorbing material (OD ₂₆₀)				Protein leakage assay (protein leakage mg/ml)				Propidium iodide uptake (RFUs)		
	0h	1h	2h	6h	0h	1h	2h	6h	1h	2h	6h
Cinnamon root powder											
1 × MIC (75 mg/ml)	0.00 ± 0.00	0.03 ± 0.01	0.05 ± 0.03	0.07 ± 0.01	0.0 ± 0.0	3.2 ± 5.5	1.3 ± 1.2	28.1 ± 3.8	288 ± 57	607 ± 77	800 ± 62
2 × MIC (150 mg/ml)	0.01 ± 0.00	0.02 ± 0.01	0.08 ± 0.02	0.12 ± 0.01	0.0 ± 0.0	7.3 ± 5.6	27.7 ± 15.5	89.7 ± 11.9	328 ± 100	912 ± 138	1086 ± 239
Peppermint oil											
1 × MIC (8% v/v)	0.00 ± 0.00	0.04 ± 0.02	0.04 ± 0.01	0.14 ± 0.04	0.0 ± 0.0	18.2 ± 7.8	21.9 ± 5.3	70.9 ± 8.4	207 ± 73	641 ± 148	985 ± 102
2 × MIC (16% v/v)	0.00 ± 0.00	0.03 ± 0.02	0.05 ± 0.01	0.13 ± 0.02	0.0 ± 0.0	28.9 ± 9.9	69.9 ± 12.3	94.0 ± 16.5	359 ± 148	906 ± 273	1107 ± 216
4 × MIC (32% v/v)	0.00 ± 0.00	0.07 ± 0.01	0.11 ± 0.01	0.24 ± 0.01	0.0 ± 0.0	36.1 ± 16.5	84.1 ± 9.9	106.2 ± 15.6	422 ± 92	867 ± 208	1074 ± 90
trans-Cinnamaldehyde											
1 × MIC (0.02% v/v)	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.04 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	125 ± 21	1166 ± 629	1474 ± 106
2 × MIC (0.04% v/v)	0.00 ± 0.00	0.03 ± 0.00	0.09 ± 0.03	0.11 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 3.5	17.7 ± 10.4	745 ± 15	1097 ± 69	1218 ± 183
4 × MIC (0.08% v/v)	0.00 ± 0.00	0.05 ± 0.02	0.12 ± 0.05	0.14 ± 0.06	0.0 ± 0.0	1.8 ± 1.9	1.3 ± 2.1	14.7 ± 7.6	618 ± 8	1458 ± 67	1264 ± 23
8 × MIC (0.16% v/v)	0.01 ± 0.01	0.08 ± 0.01	0.11 ± 0.02	0.15 ± 0.05	0.0 ± 0.0	32.1 ± 15.8	84.9 ± 17.6	86.0 ± 21.1	1369 ± 107	1719 ± 530	2688 ± 867
Menthol											
1 × MIC (9.4 mg/ml)	0.00 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	185 ± 7	230 ± 14	353 ± 90
2 × MIC (18.8 mg/ml)	0.00 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.0 ± 0.0	29.9 ± 12.4	46.8 ± 11.1	78.1 ± 24.4	285 ± 128	344.0 ± 2.9	799 ± 26
4 × MIC (37.5 mg/ml)	0.00 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.12 ± 0.07	0.0 ± 0.0	27.9 ± 14.9	63.8 ± 27.6	93.4 ± 22.2	346 ± 9	290.6 ± 73.5	844 ± 27
8 × MIC (75 mg/ml)	0.00 ± 0.00	0.04 ± 0.02	0.08 ± 0.05	0.16 ± 0.07	0.0 ± 0.0	60.8 ± 21.2	78.7 ± 21.2	91.5 ± 23.8	400 ± 125	635.1 ± 55.8	874 ± 148

Table 6.5 (Cont.) Effect of products on membrane integrity of *C. difficile* ATCC 700057, measured by an increase in extracellular 260nm absorbing materials, protein leakage and propidium iodide uptake assays.

Treatment	Extracellular 260nm absorbing material (OD ₂₆₀)				Protein leakage assay (protein leakage mg/ml)				Propidium iodide uptake (RFUs)		
	0h	1h	2h	6h	0h	1h	2h	6h	1h	2h	6h
Zingerone											
1 × MIC (9.4 mg/ml)	0.00 ± 0.01	0.07 ± 0.03	0.07 ± 0.00	0.12 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 4.9	9.8 ± 8.8	769 ± 190	632 ± 22	1777 ± 337
2 × MIC (18.8 mg/ml)	0.00 ± 0.00	0.04 ± 0.02	0.07 ± 0.01	0.14 ± 0.00	0.0 ± 0.0	8.0 ± 6.6	42.4 ± 13.6	58.1 ± 18.6	438 ± 48	786 ± 265	1569 ± 133
4 × MIC (37.5 mg/ml)	0.01 ± 0.00	0.02 ± 0.01	0.08 ± 0.04	0.15 ± 0.07	0.0 ± 0.0	29.3 ± 5.7	45.7 ± 17.2	66.8 ± 4.6	745 ± 26	1247 ± 29	1321 ± 45
8 × MIC (75 mg/ml)	-0.00 ± 0.00	0.05 ± 0.04	0.15 ± 0.04	0.27 ± 0.05	0.0 ± 0.0	27.1 ± 6.9	67.2 ± 9.4	83.0 ± 12.1	799 ± 82	1371 ± 284	2761 ± 75
Nisin (4 µg/ml)	0.01 ± 0.00	0.04 ± 0.02	0.12 ± 0.01	0.2 ± 0.04	0.0 ± 0.0	7.7 ± 5.5	37.6 ± 10.2	95.5 ± 11.4	1572 ± 456	2068 ± 334	2896 ± 847
Vancomycin (8 µg/ml)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	2.4 ± 3.1	10.3 ± 5.4	137 ± 26	223 ± 98	516 ± 209
SDW	-0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.03 ± 0.03	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 1.6	4.2 ± 4.9	111 ± 23	108 ± 44	204 ± 31
DMSO (2.5% v/v)	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.03	0.01 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 4.9	120 ± 41	116 ± 30	197 ± 48

SDW, sterile distilled water; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; RFUs: relative fluorescence units.

The OD₆₂₀s for treated and untreated *C. difficile* cells were expressed as a proportion of time zero (% t_n/t₀).

Values in bold differ significantly from controls (SDW and DMSO), *P* < 0.05.

vancomycin (8 µg/ml) did not result in any protein leakage throughout the assay.

6.2.4.3. Propidium iodide uptake assay

Nisin treatment of both *C. difficile* strains resulted in propidium iodide uptake after 1 h of exposure, indicating a decrease in membrane integrity. *C. difficile* NCTC 13366 treated with nisin showed a relative fluorescence unit (RFU) of 1448 ± 59 , compared with a value of 147 ± 26 for untreated control cells (SDW) ($P < 0.001$). Similar results were observed with nisin-treated *C. difficile* ATCC 700057 cells, with an RFU of 1572 ± 456 compared to an RFU of 111 ± 23 for the untreated control. Treatment with zingerone and *trans*-cinnamaldehyde at $\geq 1 \times$ MIC resulted in significant propidium iodide uptake by *C. difficile* cells after 1 h. A significant increase in propidium iodide fluorescence was observed for all products at most tested concentrations after 6 h of exposure. Neither *C. difficile* strains showed any evidence of cell leakage or increased permeability after treatment with vancomycin ($P \geq 0.816$).

6.2.5 Inhibition of protein synthesis

An *in-vitro* transcription/translation experiment was conducted using the *E. coli* S30 extract system for circular DNA to determine whether any products inhibited protein synthesis. Products with bacteriostatic activity against *C. difficile* (allicin, fresh garlic extract and *Leptospermum* honey (MGO 514+)) were tested at various concentrations (Table 6.6). *Leptospermum* honey (MGO 514+) was the only product to show a reduction in RLU ratio compared to untreated controls (SDW and DMSO) with values ranging from 42.1% to 68.9%. The two positive controls, streptomycin sulfate and tetracycline, both showed significant inhibition of protein synthesis/prokaryotic translation.

Table 6.6. Effect of products on inhibition of protein synthesis using the *Escherichia coli* S30 extract system.

Test products	Test concentration	RLU ratio % (mean $\pm$ SD)
Natural Products		
Fresh garlic extract	50% v/v	79.9 $\pm$ 14.5
	25% v/v	89.6 $\pm$ 6.4
	12.5% v/v	101.5 $\pm$ 5.6
Allicin	75 mg/ml	102.2 $\pm$ 5.4
	37.5 mg/ml	101.5 $\pm$ 3.1
	18.8 mg/ml	103.7 $\pm$ 2.2
<i>Leptospermum</i> honey (MGO 514+)	32% w/v	42.1 $\pm$ 11.8
	16% w/v	61.2 $\pm$ 7.3
	8% w/v	68.9 $\pm$ 6.6
Controls		
Streptomycin	1 mg/ml	0.4 $\pm$ 0.4
	0.1 mg/ml	0.6 $\pm$ 0.4
	0.01 mg/ml	6.4 $\pm$ 3.5
Tetracycline	1 mg/ml	1.6 $\pm$ 1.4
	0.1 mg/ml	49.5 $\pm$ 4.1
	0.01 mg/ml	91.6 $\pm$ 6.4
SDW		100 $\pm$ 0.0
DMSO	2.5% v/v	102.4 $\pm$ 4.1

RLU, relative light unit; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide. Values in bold differ significantly from controls (SDW and DMSO).

6.2.6 Detection of antimicrobial cross-resistance

In this experiment, the MICs of five products with bactericidal activity and four with bacteriostatic activity against *C. difficile* were determined against a panel of *C. difficile* strains showing combinations of fluoroquinolone resistance (FQR), tetracycline resistance (tetR) and macrolide-lincosamide-streptogramin B (MLS_B) resistance or susceptibility (Table 6.7). The *in-vitro* activity of the nine products is shown in Table 6.8. None of the products showed >2-fold difference in MIC between the antimicrobial resistant (FQR⁺, tetR⁺ and MLS_B⁺) and susceptible (FQR⁻, tetR⁻ and MLS_B⁻) strains, indicating a lack of cross-resistance between test substances. The attempt to generate metronidazole or vancomycin resistance strains of *C. difficile* ATCC 700057 and NCTC 13366 by serial passage resulted in increases in MIC from 0.5 µg/ml to 4 µg/ml after 20 serial passages for both antimicrobials. However, no increase in MIC for natural products was shown between the parent strains and those that had been serially-passaged against metronidazole and vancomycin (Table 6.9).

6.3 DISCUSSION

The rapid development of AMR, failure to prevent recurrences and the high cost of the current antimicrobial therapy for CDI is all serious issues²⁷⁵⁻²⁷⁷. Therefore, there is a need for better therapeutic options to effectively manage disease. Furthermore, understanding the mechanisms of action of novel antimicrobial agents and determining their target sites within *C. difficile* cells can enhance their clinical application¹⁷⁵. In this chapter, the modes of action of five natural products with bactericidal activity and four with bacteriostatic activity against *C. difficile* were investigated.

Table 6.7 Modes of action of selected antimicrobial agents.

Compound	Mechanism of action
Metronidazole	Inhibits nucleic acid synthesis by disrupting the DNA of microbial cells.
Vancomycin	Mainly acts by prevention of cell-wall biosynthesis of bacteria.
Clindamycin	Inhibits bacterial protein synthesis by inhibiting ribosomal translocation.
Tetracycline	Inhibits prokaryotic aminoacyl-tRNA binding to the ribosome small subunit (protein synthesis inhibitor).
Trimethoprim	Binds to dihydrofolate reductase and inhibits the reduction of dihydrofolic acid to tetrahydrofolic acid (inhibits bacterial DNA synthesis).
Erythromycin	Inhibits protein synthesis by binding to the 23S rRNA molecule (in the 50S subunit) of the bacterial ribosome, blocking the exit of the growing peptide chain.
Moxifloxacin	Inhibits DNA gyrase, a type II topoisomerase, and topoisomerase IV, enzymes necessary to separate bacterial DNA, thereby inhibiting cell replication.
Carbapenem	Catalyzes the formation of peptidoglycan in the cell wall of bacteria

Table 6.8 MIC values of natural products against antimicrobial resistant and susceptible *C. difficile* strains.

Isolate ID	Phenotype	Treatment								
		Fresh garlic extract (% v/v)	Garlic clove powder (mg/ml)	Allicin (mg/ml)	<i>Leptospermum</i> honey (MGO 514+) (% w/v)	Peppermint oil (% v/v)	Cinnamon root powder (mg/ml)	Menthol (mg/ml)	Zingerone (mg/ml)	<i>trans</i> -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

MGO, methylglyoxal; MLS_B, macrolide-lincosamide-streptogramin-B; tetR, tetracycline resistant; FQR, fluoroquinolone resistant.

Table 6.9 MIC values for products against metronidazole and vancomycin tolerant and intolerant *C. difficile*.

Strain ID	VAN (µg/ml)	MET (µg/ml)	Treatment								
			Fresh garlic extract (% v/v)	Garlic clove powder (mg/ml)	Allicin (mg/ml)	<i>Leptospermum</i> honey (MGO 514+) (% w/v)	Peppermint oil (% v/v)	Cinnamon root powder (mg/ml)	Menthol (mg/ml)	Zingerone (mg/ml)	<i>trans</i> - Cinnamaldehyde (% v/v)
NCTC 13366	0.5	0.5	0.8	9.4	9.4	8	8	75	9.4	9.4	0.03
NCTC 13366	4	0.5	0.8	9.4	9.4	8	4	75	9.4	9.4	0.02
NCTC 13366	0.5	4	0.4	4.7	9.4	8	4	75	9.4	9.4	0.02
ATCC 700057	0.5	0.5	0.8	9.4	9.4	16	8	75	9.4	9.4	0.03
ATCC 700057	4	0.5	0.8	9.4	9.4	16	8	75	9.4	9.4	0.02
ATCC 700057	0.5	4	0.4	9.4	4.7	8	8	75	9.4	9.4	0.01

VAN, vancomycin; MET, metronidazole; MGO, methylglyoxal.

6.3.1 Loss of cell viability confirmed by time-kill and ATP-leakage assays

Time-kill kinetics for the five bactericidal products were investigated to further understand their mode of action against *C. difficile*. Treatment with cinnamon root powder and peppermint oil, as well as the three pure compounds, *trans*-cinnamaldehyde, menthol and zingerone, resulted in $>3 \log_{10}$ reductions in *C. difficile* viable counts following 24 h of exposure, which further demonstrated the bactericidal activity of these products. Three products; cinnamon, menthol, and zingerone showed a concentration-dependent mode of killing, while killing kinetics for the two other products, peppermint oil and *trans*-cinnamaldehyde, showed a less obvious trend. Additionally, the levels of intracellular and extracellular ATP after exposure of log-phase *C. difficile* to treatments showed depletion of cellular ATP, which further confirmed their bactericidal effect against *C. difficile*. To the best of our knowledge, no published studies have previously investigated the mechanism of action including the time-kill kinetics for natural products against *C. difficile*. However, in 2015 Alam *et al.* assessed the mode of action and bactericidal properties of surotomycin as a membrane-active compound against *C. difficile*²⁷⁸. Concentration-dependent killing of both logarithmic- and stationary-phase cultures of *C. difficile* was reported for surotomycin, killing 99% of the cells at $16 \times \text{MIC}$ following 24 h of exposure (4 $\mu\text{g/ml}$)²⁷⁸.

Another study investigated the bactericidal activities of several membrane-active agents including nigericin, valinomycin, clofazimine and daptomycin¹⁷⁵. Most membrane-active agents and to a lesser extent daptomycin showed concentration-dependent killing of both log-and stationary-phase *C. difficile*. Moreover, exposure of *C. difficile* cells to the membrane-active compounds at bactericidal concentrations resulted in a rapid reduction in cellular ATP¹⁷⁵. In that study, nisin, metronidazole and vancomycin were used as controls and their effects on the ATP synthesis were consistent with the current findings. In the current study, nisin and metronidazole significantly reduced intracellular

ATP ($P \leq 0.01$), while vancomycin showed no significant effect on ATP leakage ($P \leq 0.119$). Corbett *et al.* examined the killing kinetics of SMT19969, a novel antimicrobial agent under development at the time of study, along with the comparator antimicrobials, fidaxomicin and vancomycin against two *C. difficile* strains, 630 and 5325 (RT 012 and 078, respectively) ²⁷⁹. SMT19969 resulted in a $>3 \log_{10}$ reduction in viable counts after 24 h of exposure with a concentration-independent pattern of killing. Meanwhile, fidaxomicin showed a time-dependent mode of killing, and as expected, vancomycin was bacteriostatic at all concentrations ²⁷⁹.

6.3.2 Cell wall and membrane studies

Damaging the cell wall can lead to cell lysis, resulting in subsequent cell death ¹⁷⁶. The essential oils from thyme, oregano and rosewood have been shown to cause lysis of *Streptococcus pneumoniae* ^{176,280}, however, in this study, no products resulted in the lysis of *C. difficile* cells. In contrast, the cell permeability assays showed that all five bactericidal products damaged the cell membrane. The cytoplasmic membrane acts as a selective barrier and is also involved in energy transduction allowing various endergonic processes ^{281,282}. Retaining membrane integrity is vital for the survival of logarithmic- and stationary-phase cells of *C. difficile*, hence the cell membrane is regarded as an important target for antimicrobial agents that may be used to treat CDI ^{175,281}.

Essential oils and their active components exert antimicrobial activity against a variety of targets, although the cytoplasmic membrane is a major target, as also observed in this current study ^{213,215,283}. As stated by Nazzaro *et al.*, *trans*-cinnamaldehyde at lethal concentrations can damage the cell membrane ²¹³. In the current study, the leakage of 260nm absorbing materials from bacterial cells indicated cytoplasmic membrane damage, and this was observed for all bactericidal products, except for cinnamon root. Nonetheless, a long exposure time, as well as a high concentration of products, were

required to show significant leakage of materials. In contrast, significant protein leakage from bacterial cells was observed after a shorter exposure time for all five products. Similarly, the propidium iodide uptake assay further proved disruption/loss of integrity in the cytoplasmic membrane of *C. difficile* cells following exposure to all the bactericidal products.

Essential oils contain hydrophobic compounds that can penetrate Gram-positive bacterial cells and act on the cytoplasmic membrane^{284,285}. Also the phenolic compounds present in essential oils may show antimicrobial activity against Gram-positive bacteria²¹³. Disturbing the membrane structure can affect other cellular functions such as alteration of membrane fluidity and leakage of cell contents^{281,284}. The alteration in membrane permeability and changes in integrity of the membrane affect its functions due to uncontrolled entry or loss of metabolites and ions, as well as entry of essential oils in the cytoplasm which will disturb the membrane-embedded enzymes^{175,213,286}.

6.3.3 Inhibition of protein synthesis as a possible mechanism of action

One of the likely mechanisms of action for bacteriostatic agents is the inhibition of protein synthesis^{287,288}, therefore, the ability of natural products to inhibit protein synthesis was investigated. Both the antimicrobials tetracycline and streptomycin have previously been shown to inhibit prokaryotic translation²⁸⁷. Streptomycin has an impact on several steps involved in protein synthesis, leading to translational errors and a decreased rate of translocation²⁸⁹. Tetracycline inhibits protein synthesis by preventing the binding of RNA to ribosomes, mainly by binding to the 30S ribosomal subunit in the mRNA translation complex²⁸⁹. In the current study, streptomycin and tetracycline served as positive controls and both showed significant inhibition of protein synthesis. Of the natural products, *Leptospermum* honey was the only product that showed a reduction in RLU ratio, indicating a likely impact on protein synthesis. In previous microarray and

proteomic studies of bacteria, an effect of *Leptospermum* honeys on the suppression of protein synthesis has been also indicated^{162,163}.

6.3.4 Lack of antimicrobial cross-resistance

Over time, *C. difficile* has developed resistance to several different classes of antimicrobials, however, the rate varies widely depending on geographic regions and antimicrobial use policy²⁹⁰. The alteration of antimicrobial targets and the presence of resistance-associated genes in the bacterial chromosome are the main factors in the emergence of AMR in *C. difficile*²⁹⁰. In order to manage the AMR crisis, coordinated efforts are required to implement new and alternative therapeutic options with less chance of acquiring resistance. In the current study, nine products were tested against a panel of *C. difficile* strains showing combinations of FQR, tetR and MLS_B resistance or susceptibility. Notably, none of the natural compounds showed higher MICs against resistant strains. This suggests that the mode of action for these natural compounds may be different from that of fluoroquinolones (targeting DNA gyrase), tetracycline (targeting riboprotein proteins) and macrolides/lincosamides (targeting 23s rRNA). In addition, none of the products showed higher MICs against metronidazole- or vancomycin-tolerized *C. difficile* isolates.

According to previous investigations, resistance to metronidazole is likely due to alterations in several metabolic pathways, mainly those involved in the activity of nitro-reductases, DNA repair and iron uptake^{291,292}. Moreover, vancomycin resistance is likely to be mediated by amino acid changes in peptidoglycan biosynthesis-associated proteins²⁹³. Regarding the development of resistance by bacteria to natural compounds, this is unlikely to occur^{216,294,295}. Blair *et al.* showed in 2009 that the serial passaging of *S. aureus* and *Pseudomonas aeruginosa* with active *Leptospermum* honey or the antimicrobials tetracycline and ciprofloxacin did not induce resistance to honey when

maintained under the same conditions that rapidly induced resistance to antimicrobials¹⁶². Therefore, compounds derived from plants may have great potential as antimicrobials against drug-resistant microorganisms.

The multiple components in a crude plant extract are likely to act at different sites, contributing to its overall activity¹⁹⁵. As mentioned before, not all natural products with antimicrobial activity kill the microorganisms, rather some affect key events in the pathogenic process²⁹⁶. Phytoactive components either alone or in combination with conventional antimicrobials might be considered as a possible approach in dealing with the global antimicrobial resistance crisis^{196,296}. It has been suggested that microbes have a reduced ability to adapt to a plant-based antimicrobial approach¹⁹⁵. A range of compounds is produced from plants which provide an adaptive capability to adverse environmental conditions^{297,298}. These biologically active organic compounds represent the secondary metabolites, which act as a defense against molecular targets in microbes^{195,299}.

6.4 CONCLUSIONS

This chapter provides a fundamental framework regarding the possible mechanisms of action of natural products against *C. difficile*. Whilst the cell wall appeared to be unaffected, several compounds produced damage to the cell membrane and induced depletion of intracellular ATP in *C. difficile*. These factors are important targets for investigating anti-*C. difficile* drugs to reduce the burden of CDI. Also, as we are unlikely to observe cross-over mechanisms of resistance between standard antimicrobials and natural compounds, it further increases the latter's potential in reducing the increasing rate of AMR.

Chapter 7

Investigating the efficacy of products with potent *in-vitro* activity in a mouse model of CDI

7.1 INTRODUCTION

C. difficile infection has been studied using various animal species as models, including hamsters, guinea pigs, rabbits, rats, mice, foals, piglets and monkeys ³⁰⁰. The hamster model is one of the most widely used animals, whereby CDI is initiated by depleting the gut microbiota by exposure to various antimicrobials ^{250,301,302}. Following challenge of the hamsters by toxigenic *C. difficile*, disease quickly develops into a fulminant and lethal stage, which does not represent the usual course of CDI in humans ^{300,301}. However, despite the limitations associated with the use of the hamster model of the disease, it has been used in the study of CDI pathophysiology and efficacy testing of potential therapeutics for nearly three decades ^{250,301-304}.

The relevance of animal models to human disease and whether infected animals present with similar pathological features to those seen in humans is of particular importance. Mouse models have been used extensively to investigate the disease mechanisms and the effect of different therapies ³⁰⁰. Conventional mice are not as readily susceptible to CDI as hamsters are, but the disease state in mice more closely resembles that found in humans ³⁰⁵. Therefore, in this chapter, a mouse model of CDI was used to evaluate the efficacy of selected compounds *in-vivo*. Considering the *in-vitro* activity of various natural products against *C. difficile* as described and studied in previous chapters, two pure compounds, *trans*-cinnamaldehyde and zingerone with potent *in-vitro* activity were chosen to be assessed for efficacy in a mouse model of CDI. The oral route of administration was selected to investigate the effect of these compounds in the treatment of *C. difficile* infected mice since it is a simple and convenient route of administration for both humans and animals ³⁰⁶.

7.1.1 Aims

The purpose of this study was to investigate the ability of two pure compounds, *trans*-cinnamaldehyde and zingerone, to treat CDI in a mouse model. The animal trial was conducted by collaborators at Monash University (Melbourne, Australia).

7.2 RESULTS

None of the mice treated with either natural products, *trans*-cinnamaldehyde or zingerone, survived the infection (Figure 7.1). All mice treated with zingerone were culled at 24 h, due to ethics requirements of reaching 10% weight loss within 24 h. Mice treated with *trans*-cinnamaldehyde showed weight loss and survival rates similar to the 10% ethanol treated control group. Uninfected mice were screened at multiple time points, with no *C. difficile* detected (Figure 7.2). The *C. difficile* spore counts in faecal samples after 24 and 48 h are shown in Figure 7.3. There was no significant protection from *C. difficile* disease observed after administration of either natural product in this trial. Mice in the infected groups were colonized to high levels, regardless of treatment type. In the spore count figures, the dotted line represents the limit of detection (Figure 7.3).

7.3 DISCUSSION

CDI, and in particular the resulting colitis in mice, reflects key components of the disease process in humans, such as involvement of the entire colon, variable disease severity, and disease recurrences after antimicrobial therapy^{305,307,308}. The development of robust mouse models has begun to provide insight into the fundamental biology of this pathogen; it also facilitates the expansion of pre-clinical models required to obtain a better understanding of the effect of novel treatments and prophylactic approaches^{300,305,307}. Due to the persistent nature of spores and limited therapeutic options, there

Figure 7.1 Kaplan-Meier survival curves comparing the survival rate of mice infected with *C. difficile* M7404 and treated with 10% ethanol (solvent), zingerone or *trans*-cinnamaldehyde, to uninfected mice.

Figure 7.2 Kaplan-Meier weight-loss curves comparing the relative weight of mice infected with *C. difficile* M7404 and treated with 10% ethanol (solvent), zingerone or *trans*-cinnamaldehyde, to uninfected mice.

Figure 7.3 The 24 and 48 h colonization of *C. difficile* spores in faecal samples in *C. difficile* M7404 infected mice treated with 10% ethanol, zingerone or *trans*-cinnamaldehyde.

are challenges involved in controlling and treating CDI³⁰⁹. The goal of this study was to investigate the effect of two pure compounds with potent *in-vitro* activity in an experimental murine model of antimicrobial-associated CDI. However, the *in-vivo* results did not reflect the success of *in-vitro* findings. Notwithstanding the theoretical similarities in the disease state between the conventional mice and humans, each has distinct anatomy and immunity. At the same time, there were several factors or limitations associated with the study that may explain why neither of the natural compounds showed any therapeutic effect against CDI. Mice are not as susceptible to CDI as hamsters, but they are more representative of the disease state in humans³⁰¹.

At 6 h post-infection, the mice received treatment by oral gavage for the first time, followed by subsequent treatments given in their drinking water with only 10% ethanol used as the solvent for the compounds. Both compounds have low solubility in water, thus in previous *in-vitro* studies, 20% DMSO was used as the primary solvent for these compounds. However, since DMSO is not palatable for animals and the mice were therefore unlikely to drink it, the best alternative was to use 10% ethanol as the solvent. However, these compounds are only partially soluble in 10% ethanol and they would have been mainly suspended as oily droplets in water. Moreover, none of the compounds or solvent have a pleasant or appealing taste or odour for mice, and as such they may not have finished their daily drinking water. For instance, *trans*-cinnamaldehyde is a yellowish oily liquid with a strong flavour and odour of cinnamon¹⁸³ and zingerone is a key component of the pungency of ginger¹⁵⁷. Therefore, even if mice were monitored daily to ensure they were drinking the compounds added to their drinking water, some of the compounds might have been left as emulsions or droplets in the water container, which means that mice might not have received the correct dosage, and it was likely reduced compared to the concentration of compounds used in the oral gavage.

Another major limitation of the study was the fact that compounds were given to mice orally without microencapsulation. Encapsulation is important for enhancing the oral bioavailability of compounds by protecting them from chemical and enzymatic attacks in the body until they reach the desired site of action^{183,310}. In addition, the amount of, and rate at which, the compounds reach the bloodstream are less predictable compared to more direct routes of administration, such as injection³⁰⁶. However, the oral route still remains a preferred mode of drug administration, while poorly water-soluble compounds can suffer from low bioavailability when administered orally³⁰⁶. The efficacy of oral drug delivery administered through microcapsules was previously investigated in several studies^{183,311}. In a study by Quenelle *et al.*, large and small microsphere formulations of rifampin were prepared and administered once and twice respectively, following initial infection of mice with *Mycobacterium tuberculosis*³¹². Another group of mice was administered every day by oral gavage with various doses of rifampin. At day 26 post-infection, the reduction in CFU was used for comparison. The microsphere formulation of rifampin could be used for treating tuberculosis with minimal dosing and sufficient levels of drug delivered in the blood through microsphere technology to treat the infection³¹². Another study investigated the toxicity of high doses of *trans*-cinnamaldehyde in mice and rats administered orally through microcapsules since microencapsulation had been recognized as an effective method for administering reactive aldehydes^{183,185}.

Treatment of CDI in mice by the use of conventional antimicrobial agents has been previously examined³¹³⁻³¹⁵. In a murine model of CDI, mice were treated with vancomycin, metronidazole, a combination of vancomycin and metronidazole or placebo for 10 days³¹³. The treatments were administered every day by oral gavage. The mice treated with either vancomycin alone or a combination of both antimicrobial

agents recovered faster than those treated only with metronidazole ³¹³. Administration of treatment in animal models through oral gavage is more reliable than adding the treatment to their daily drinking water and monitoring their consumption.

A limited number of natural compounds has been examined as possible alternative prophylactic or therapeutic options in animal models of CDI ³⁰⁹. Carvacrol is a monoterpenoid phenol naturally present in oregano and thyme oil. The *in-vitro* inhibitory effect of carvacrol on *C. difficile* toxin production without affecting the gut microbiota was previously demonstrated, which led the further investigation of this compound in an *in-vivo* model ³⁰⁹. Five to six week old mice were randomly assigned to various treatment groups of eight mice each. Mice were given powdered feed supplemented with 0, 0.05 and 0.1% carvacrol, then infected with *C. difficile* spores. Carvacrol supplementation reduced the severity of clinical infection with minimal effect on gut microbiota diversity ³⁰⁹.

Neither zingerone nor *trans*-cinnamaldehyde have been investigated for the treatment of CDI in an *in-vivo* model. However, the prophylactic activity of *trans*-cinnamaldehyde in preventing uropathogenic *E. coli* (UPEC) in a mouse model was evaluated ¹⁸⁴. The *trans*-cinnamaldehyde containing mouse chow showed a significant reduction in UPEC colonization in the urethra and bladder compared to the control group ($P \leq 0.05$) ¹⁸⁴. Mouse models have also been used to examine the effect of *trans*-cinnamaldehyde on the cardiovascular and digestive systems ³¹⁶. The study showed moderate inhibition of the mouse intestinal propulsion when *trans*-cinnamaldehyde was administered intravenously at 5 and 10 mg/kg body weight and there was a stimulating effect on the cardiovascular system ³¹⁶. The improvement on memory impairment in the mice challenged with lipopolysaccharide was observed by intraperitoneal injection of *trans*-cinnamaldehyde (1.25-20 μ M) ¹⁸². On the other hand, the protective effect of zingerone against inflammation by lipopolysaccharide induced from liver injury during *P.*

aeruginosa infection was explored in a mouse model ³¹⁷. In this study, zingerone showed significant anti-inflammatory activity when administered intraperitoneally (100 mg/kg body weight) ³¹⁷. Another study was undertaken to elucidate the radioprotective and antigenotoxic effect of zingerone in mice exposed to whole body gamma irradiation ¹⁸⁸. Zingerone (20 mg/kg body weight, orally administrated) mitigated radiation-induced cytogenetic damage as well as animal mortality ¹⁸⁸.

7.4 CONCLUSION

Both *trans*-cinnamaldehyde and zingerone are inexpensive dietary natural compounds which are known for their potent medicinal effect for centuries. The *in-vivo* study using a mouse model of CDI did not support the results observed with these compounds *in-vitro*. However, there were several limitations of the mouse study and therefore, further investigation of these compounds with alternative and optimized routes of administration and more efficient techniques are essential to be able to more confidently evaluate the efficacy of these compounds.

Chapter 8

General discussion

8.1 INTRODUCTION

C. difficile is a major cause of antimicrobial- and healthcare-associated diarrhoea and its incidence and severity appear to be on the rise ³⁷. Uptake of the organism through ingestion does not necessarily cause infection, due to the protective effect of the colonic flora which usually resists colonization and overgrowth of *C. difficile* ³¹⁸. However, if the gut microbiota is disturbed, acquisition of the organism from an exogenous source can induce disease under certain conditions ³¹⁸. Antimicrobial exposure is considered to be the main risk factor for CDI and several studies have shown the association between recent antimicrobial exposure and CDI ^{37,319,320}. Although hospitalized elderly patients receiving antimicrobial therapy remain the group most at risk, CDI has been increasingly observed in a younger population with no previous exposure to healthcare settings or antimicrobials ³⁷.

The emergence of hyper-virulent *C. difficile* strains, an aging population, and pervasive antimicrobial overuse have resulted in increased infection rates and poor clinical outcomes ^{321,322}. Fifteen to 25 years ago, there was little interest in developing new and alternative therapeutic options for the treatment of CDI, as most patients with CDI responded to conventional antimicrobials and severe cases of infection were less frequent ³²³. However, during the past 15 years, the limitations of conventional and current therapies have become more apparent due to the relative lack of efficacy, multiple dosing requirements, development of resistance and their high cost ³¹⁹.

Around 50% of currently available drugs have been sourced originally from natural products and their derivatives ^{322,324}. Historically, plant extracts have formed the basis for development of new and novel therapeutics ^{197,325}. Natural products have been extensively used as the main source of treatment in countries such as China and India ³²². Plant extracts are effective in the treatment of several infectious diseases ³²⁵⁻³²⁷. Some plant-derived remedies that are targeted towards infectious diseases include tea tree oil,

cranberry juice and honey ³²⁶. For instance, tea tree oil has broad-spectrum antibacterial activity *in-vitro* especially against MRSA. It has also demonstrated both antifungal and antiviral activities *in-vitro* and *in-vivo* ^{328,329}. These properties suggest that tea tree oil may be useful in treating oral candidiasis and acne as well as decolonising MRSA carriage ^{176,326,328-330}. Various medicinal plants and their secondary metabolites are self-administered for treating gastrointestinal disorders and diarrhoea-associated diseases ³²⁴. Investigating approaches primarily used in treating other toxin-mediated diseases that share similarities with CDI may be worthwhile. Therefore, the overall aim of this study was to investigate the *in-vitro* and *in-vivo* activity of natural products against *C. difficile* using several different approaches.

8.2 KEY FINDINGS

8.2.1 Antimicrobial effect of natural products on *C. difficile* and gastrointestinal microbiota *in-vitro*

Fourteen out of 22 products showed some degree of antimicrobial activity against all four *C. difficile* strains, as described in Chapter 3. While nine of those products showed bacteriostatic activity, the remaining five had a bactericidal effect. In addition, the gut organism *L. casei* showed less susceptibility to most of the products compared to *C. difficile* and *Bacteroides*. Thus, this was promising in regard to the potential use of natural products as either supplementary or alternative options, as they may be less disruptive to the commensal gut organisms such as Lactobacilli.

The natural products derived from medicinal plants are a rich source of biologically active compounds ^{131,132}. The antimicrobial activity of some of these products is partly due to their antioxidant and immunomodulatory properties, stimulating both specific and non-specific immunity ¹³¹. Several factors can influence the antimicrobial activity of natural products such as botanical source and method of extraction, as well as the composition

and structure of natural extracts ^{131,271}. One of the main steps towards discovering antimicrobial drugs is the screening of natural products for antimicrobial activity ^{10,131}. In the current study, an agar well-diffusion assay was used for preliminary antimicrobial screening. This method is simple, practical and of low cost, and the results are easily interpretable ³³¹. These factors all contribute to the common use of this technique for antimicrobial screening of plant extracts, essential oils, and other antimicrobials ³³¹. Further susceptibility testing of the products was performed using a broth microdilution assay. Dilution methods are considered as reference methods for performing susceptibility testing *in-vitro* and are frequently used in evaluating the performance of other susceptibility testing techniques ^{331,332}.

8.2.2 Combination therapy between synthetic and naturally occurring antimicrobials

The antimicrobial activity of natural products was examined in Chapter 3, as well as their interactions with the common antimicrobials, metronidazole, and vancomycin. Use of appropriate *in-vitro* testing is crucial for the accurate prediction of synergy between drugs ^{333,334}. In this study, checkerboard method, which is one of the most widely used techniques, was used to detect synergy between commercial drugs and natural products. Synergistic activity was observed between *trans*-cinnamaldehyde and vancomycin with partial synergy observed between *trans*-cinnamaldehyde and metronidazole. There was no contraindication or antagonism between the antimicrobials and natural products, which encourages the further investigation of natural extracts in combination therapy to overcome the development of resistance.

Mechanisms contributing to AMR include the destruction of antimicrobials via enzymes produced from bacteria (such as beta-lactamases), modification of active site on the target and efflux of antimicrobial from the cell ³²¹. AMR to existing drugs is steadily on the rise,

hence there is a need to search for new and alternative classes of antimicrobial substances especially those from natural sources ^{321,326}. Sometimes, the desired effective outcomes are not achieved by the use of single antimicrobials and a combination of drugs may exceed their individual performance ³³⁴. Benefits associated with the synergistic interactions are greater efficacy; better stability or bioavailability of free agents, less adverse effects and achievement of adequate therapeutic effects with relatively small doses ³³³⁻³³⁵.

Plant-derived antimicrobials have been suggested as synergistic enhancers that, when taken concurrently with conventional drugs, may enhance the drug's activity ³³⁴. In particular, essential oils are complex, multi-component substances that consist mainly of terpenes as well as some non-terpene components ³³³. Essential oils exert different modes of action to inhibit pathogens, with one frequent mechanism being the partitioning of components into the lipid bilayer of the cell membrane, facilitating the uptake of antimicrobials, resulting in leakage of essential cell contents ³³³.

8.2.3 Impact of natural products on sporulation and vegetative outgrowth of *C. difficile*

During CDI, a sporulation process is initiated, resulting in dormant spores that persist in the host and disseminate in the environment ^{226,336}. The mechanisms involved in *C. difficile* sporulation have not been extensively studied, while the mechanisms involved in formation of *B. subtilis* spores have been studied in greater detail ³³⁶. However, since several regulatory factors that direct gene expression specific to compartments such as RNA polymerase sigma factors are highly conserved in the two species, knowledge derived from *B. subtilis* helps us to understand the complex process involved in spore formation of *C. difficile* ³³⁶.

Spores are pivotal to disease transmission and the infectious cycle as the strains that are unable to form endospores, will not survive in the colonic environment of the host ²²⁶. Therefore, strains with increased sporulation proficiency are a particular health concern. *C. difficile* forms spores in order to avoid the toxic effect of oxygen and to survive transition to a new host ^{222,336}. During sporulation two subcellular compartments coordinate to carry out the cellular morphogenesis resulting in the production of dormant spores ²²⁶. In 20%-25% of cases of infection relapse, spores are implicated; it is suggested that spores remain in the intestinal crypts and, once antimicrobial treatment is completed, they promote renewed or recurrent disease ³³⁶. Thus, treatments that inhibit spore formation, can reduce the risk of transmission and recurrences ¹⁶⁸. As shown in Chapter 4, an investigation into the effect of natural products on the different stages of the spore cycle of *C. difficile* showed that several products, such as onion bulb extract and coconut oil reduced spore formation in four different *C. difficile* strains. A transcriptomic study in *C. difficile*-colonized mice has shown that genes associated with transcription of sporulation were upregulated as early as 8 h post-infection, suggesting that sporulation occurs early *in-vivo*. Thus, treatments with sporulation inhibition potency are recommended to be taken early after infection to reduce the risk of recurrence ¹⁶⁸.

The effects of products on spore germination and vegetative outgrowth of *C. difficile* were explained and studied in Chapter 4. The mechanisms involved in the germination of *C. difficile* spores are less well-studied due to low levels of homologues of proteins in the spore coat compared to other spore-forming bacteria such as *Bacillus* species ²²⁴. Spore germination is regulated by the Csp family of (pseudo) proteases and the cortex hydrolase SleC ³³⁷. The bile salt germinant receptor, CspC activates protease CspB that activates SleC lytic enzyme. The active SleC alters the protective cortex layer, triggering outgrowth of *C. difficile* ^{224,337}. Despite no effect on spore germination of *C. difficile* being observed

in the present study, several products inhibited the vegetative outgrowth of *C. difficile*. Once the ingested spores reach the anaerobic environment of the gut, they germinate to produce vegetative cells that, in susceptible individuals, may result in diarrhoeal disease via toxin production ²⁴⁰. Using treatments that prevent the outgrowth of vegetative cells would subsequently inhibit toxin production and reduce other adverse effects.

8.2.4 Anti-virulence activity of natural products against *C. difficile*

Clostridial toxins are of major importance in the pathogenesis of CDI and these have been a focus of investigation ³²⁰. The targets for toxins TcdA and TcdB are the RHO and RAC GTPases in the cytosol of host cells, which are involved in vital cellular processes such as cell to cell adhesion and maintenance of the cytoskeleton. Toxins disrupt the tight junctions and impair the intestinal barrier function leading to fluid accumulation and intestinal damage ³²⁰. Toxins also trigger the release of pro-inflammatory cytokines from epithelial and mucosal cells resulting in neutrophil influx and further damage to the intestinal lining.

Considering the key role of toxins in the initiation and pathogenesis of the infection, targeting their production or activity has been a major focus in the development of new and alternative strategies in the prevention and treatment of CDI ³³⁸. The effect of natural products against toxin production and activity of *C. difficile* was investigated and described in Chapter 5. As discussed previously, several natural products had activity against *C. difficile* toxins *in-vitro*. These data encouraged further investigation in plant-derived products as potential anti-virulence approaches in controlling CDI.

A number of antimicrobials such as tigecycline and fidaxomicin suppress toxin production ^{270,339}, yet the high cost of these agents limit their use. Several non-antimicrobial approaches with activity against *C. difficile* toxins have been previously studied and developed ^{1,59}. For instance, there are different types of adsorbents including

ion exchange resins (e.g. cholestyramine) and polymers (e.g. tolevamer) but their low therapeutic efficacy has meant that their use is either limited or has been discontinued ⁵⁹. Another approach in the prevention of CDI is the development of an effective vaccine, able to elicit satisfactory serum antibody responses in healthy adults, which would contain the combined recombinant peptides targeting both *C. difficile* toxins ^{64,340}. Again there are challenges involved with *C. difficile* vaccine. The production of these agents is expensive and both preclinical and clinical testings are required during the manufacturing period for compliance with quality standards ⁶⁴. Also, they have to be administered before the onset of disease. Whilst they are a treatment rather than a prevention, natural occurring antimicrobials have the advantage of having a comparably cheap cost of production and they can be formulated to be orally bioavailable.

8.2.5 Natural products show diverse mechanisms of action

Chapter 6 provides a framework for the mechanisms of action of various natural products that showed either bacteriostatic or bactericidal activity against *C. difficile*. Natural products have a range of diverse and multi-dimensional chemical structures ^{283,327}; several compounds derived from plants such as phenols, quinones, flavones, and terpenoids exert antimicrobial activity ^{338,341}. Time-kill assays are one of the most appropriate methods for determining bactericidal effects, and the results provide information about the dynamic interaction between antimicrobials and microbes ³³¹. Time-kill assays can indicate time-dependent or concentration-dependent antimicrobial activity ³³¹. All five products determined to have bactericidal activity based on MBC values were further investigated in a time-kill assay and most showed a concentration-dependent effect against *C. difficile*. Among the bactericidal products investigated in this study, several were essential oils or their constituents such as peppermint oil and menthol. Essential oils are mixtures of volatile secondary metabolites extracted from plants ^{342,343}. The mechanisms of action of

essential oils against microorganisms are complex but depend on their hydrophilic or lipophilic character^{282,341}. The oil components are speculated to destroy the cytoplasmic membrane resulting in the leakage of cytoplasmic contents³⁴¹. Essential oils have also been known to inhibit the synthesis of DNA, RNA, and proteins in bacterial cells^{282,342}. However, in the current study, no lysis of bacterial cells was observed to demonstrate the hypothesized/suspected effect on the cell wall, while the damage to the clostridial cell membrane was demonstrated in several assays quantifying alterations in cell permeability.

The cytoplasmic membrane contains about one-third of the total proteins of a prokaryotic cell, making it a site for vital processes such as active transport of nutrients and wastes²⁸⁶. The bacterial cell membrane also provides a selective permeability barrier and regulates cellular homeostasis and metabolic energy-transduction. Membrane-active agents can interact with multiple targets in the membrane and thus eliminate the ability of bacteria to acquire resistance^{197,286}. This phenomenon was also demonstrated in this study, as none of the natural products showed increased MICs against antimicrobial-resistant *C. difficile* strains.

The likely superiority of bactericidal agents to bacteriostatic agents is of little relevance when considering the treatment of the majority of infections caused by Gram-positive bacteria³⁴⁴. For instance, meningitis is commonly treated with bactericidal agents, however bacteriostatic agents including chloramphenicol and linezolid have been successful in treatment of this infection³⁴⁴. Information on the potential action of antimicrobial agents such as their *in-vitro* bacteriostatic or bactericidal activity is only one of the several factors important in determining a satisfactory clinical outcome^{344,345}. Also, the clinical outcome must be reflected as the major guide for the treatment of infections. Among the products showing bacteriostatic activity against *C. difficile*, including garlic preparations, allicin and *Leptospermum* honey, only honey showed

significant inhibition of protein synthesis in an S30 *E. coli* extract system. As previously reported, the antimicrobial activity of garlic, and its main constituent allicin is speculated to be due to inactivation of extracellular enzymes through cleavage of disulfide bonds and formation of reactive thiocyanate radicals ¹⁴¹.

8.3 DEVELOPMENT OF NATURAL PRODUCTS AS *C. DIFFICILE* TREATMENTS

Natural products have a range of biological activities which may be effective in managing infectious diseases ¹⁹⁵. The current treatment options for CDI are relatively expensive and may promote AMR ^{195,271}. Thus, research exploring natural products for development of novel compounds for treatment of CDI is needed.

The favorable effects of natural products in treating diarrhoeal diseases predominantly caused by *E. coli* have been previously shown ^{144,156}. Harmless strains of *E. coli* naturally exists as part of gut microbiota, however, other strains of this organism are pathogenic to humans and cause enteric diseases ⁵⁷. There are also studies describing the antimicrobial activity of natural extracts against *Helicobacter pylori* that is usually found in the stomach ³⁴⁶⁻³⁴⁸. Due to the similarities shared between these organisms, similar therapeutic agents may work effectively against all. Honey and garlic have been reported to exhibit *in-vitro* antimicrobial activity against both *E. coli* and *H. pylori* ^{141,158,346,347,349}. Research conducted on *trans*-cinnamaldehyde has demonstrated the effective prevention of uropathogenic *E. coli* biofilm *in-vitro* ³⁵⁰ as well as reduction of uropathogenic *E. coli* colonization in mice ¹⁸⁴. In addition, zingerone as mentioned in Chapter 5, has been reported to inhibit enterotoxigenic *E. coli* toxin-induced diarrhoea in mice ¹⁵⁶.

The toxicity risk of natural remedies needs to be taken into consideration and moderation in their consumption is recommended ³⁵¹. It has been suggested that encapsulation may reduce the toxicity, if any, of plant extracts ¹⁹⁵. Also long-term trials of reasonable

duration may indicate possible side effects of different natural extracts^{195,348}. Increasing our knowledge of the therapeutic effects and safety of natural products as well as improvement of experimental and clinical research plans may be useful in understanding the possible potency of these products either on their own or as adjuvant therapy for treating CDI or other gastrointestinal disorders.

8.4 STUDY LIMITATIONS AND DIRECTIONS FOR FUTURE STUDIES

There were a number of limitations in this study that needs to be addressed. First, there are differences in the immune state and anatomy between mice and humans, and the response to treatment in an animal model does not necessarily represent the effect in humans. Moreover, despite their similarities, most of the products and compounds used in this work were either only partially, or completely insoluble in water. Thus an appropriate solvent was required for dissolving the products in most experiments. It was decided that 20% DMSO would be used as the primary solvent for the products and further dilution was performed in SDW. A higher DMSO concentration could not be used as the primary solvent, as it would have interfered with bacterial growth. Therefore, some of the products may not have been completely soluble in 20% DMSO and their effect and activity may have been underestimated in some instances. Although some of the active components were tested in this study, no chemical analysis of the natural products was performed to determine their chemical composition. Therefore, until such studies are done, the results obtained from the investigation of these products need to be interpreted with caution.

Also, as mentioned in Chapter 3, there are inherent advantages as well as limitations associated with each susceptibility test. For instance, an agar well diffusion which was used as the screening method in this work does not give an MIC value and some of the extracts diffused poorly through the agar medium²⁰¹. Instead, results of the broth

microdilution assay provided more quantitative results, such as MIC values. The MIC values do not distinguish between bactericidal and bacteriostatic agents and further testing such as determining MBC values and using a time-kill assay were required to give an indication of the mode of action ^{201,332}. Therefore, several different types of susceptibility tests need to be performed to overcome the drawback of each testing technique and to gain more understanding about the possible applications of different agents.

Another limitation of this work was evident in the animal study described in Chapter 7. The *in-vivo* study did not show the expected efficacy of two products with potent *in-vitro* activity. This was possibly due to the route of drug administration. As discussed previously, the two chosen compounds had low solubility in water and they were given to infected mice in their drinking bowl using only 10% ethanol as the solvent. A suggested study improvement would be microencapsulation, which may have increased the bioavailability of the compounds but this was not possible in the current study ¹⁸³.

Therefore, potential future studies resulting from this research include:

- Further studies on the chemical composition of these products.
- Additional studies on the molecular basis of synergistic interactions, to understand the synergistic mechanisms which are fundamental to the development of pharmacological agents using medicinal plants in treating infections.
- Exploring more medicinal plants which exhibit synergistic interactions with antimicrobials.
- Further work investigating the mechanistic basis for anti-toxin activity of products.
- Determining the effect of products on transcription of genes in the PaLoc region gene.

- Molecular and metabolomics studies further investigating the mechanisms of action of products with promising *in-vitro* activity.
- Further animal trials with a better route of administration and more efficient techniques to evaluate the *in-vivo* efficacy of potent natural compounds.

8.5 CONCLUDING REMARKS

C. difficile is a multidrug-resistant pathogen present in humans, animals and the environments. CDI is a major threat in hospitalized patients and is increasingly identified in the community. It is essential to accurately diagnose this infection in order to ideally treat and prevent disease. Natural products are too valuable to be disregarded in the development of new, alternative and complementary therapeutic agents. The accessibility, convenience and general acceptability of natural products, as well as their low cost, are contributing factors towards increasing investigation of their medicinal effect. This thesis provides a fundamental framework regarding the effects of natural products on different aspects of *C. difficile* and CDI focussing on *in-vitro* effects but including an insight into *in-vivo* activity. The results suggest that several natural products including plant extracts and food-grade products may have the potential to be considered as a possible alternative or complementary treatment options for CDI. Future work is encouraged to further expand our knowledge and understanding in this field.

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APPENDICES***Contents***

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Appendix I Supplementary data Chapter 4

Supplementary Table 1A The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain NCTC 13366.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh garlic bulb (50% v/v)	1 ± 0	0.969 ± 0.013	0.956 ± 0.009	0.948 ± 0.008	0.943 ± 0.007	0.941 ± 0.011	0.941 ± 0.011	0.928 ± 0.012	0.945 ± 0.012	0.962 ± 0.014	1.007 ± 0.015	1.030 ± 0.006	1.038 ± 0.016	1.155 ± 0.010	1.166 ± 0.019	1.191 ± 0.02	1.349 ± 0.038
Fresh ginger rhizome (50% v/v)	1 ± 0	0.988 ± 0.016	0.976 ± 0.006	0.972 ± 0.007	0.968 ± 0.009	0.973 ± 0.013	0.983 ± 0.012	0.986 ± 0.014	1.008 ± 0.015	1.012 ± 0.015	1.031 ± 0.014	1.040 ± 0.013	1.050 ± 0.015	1.061 ± 0.016	1.066 ± 0.017	1.072 ± 0.017	1.273 ± 0.034
Fresh onion bulb (50% v/v)	1 ± 0	0.977 ± 0.011	0.960 ± 0.016	0.949 ± 0.012	0.962 ± 0.015	0.960 ± 0.023	0.975 ± 0.026	0.977 ± 0.030	0.979 ± 0.016	0.979 ± 0.011	0.986 ± 0.037	1.006 ± 0.028	1.010 ± 0.031	1.017 ± 0.029	1.023 ± 0.016	1.025 ± 0.029	1.014 ± 0.076
<i>Leptospermum</i> honey A (32% w/v)	1 ± 0	0.981 ± 0.004	0.963 ± 0.004	0.949 ± 0.015	0.935 ± 0.014	0.929 ± 0.011	0.919 ± 0.004	0.914 ± 0.020	0.905 ± 0.010	0.902 ± 0.012	0.898 ± 0.013	0.904 ± 0.019	0.909 ± 0.022	0.907 ± 0.014	0.899 ± 0.015	0.893 ± 0.019	0.865 ± 0.022
<i>Leptospermum</i> honey B (32% w/v)	1 ± 0	0.985 ± 0.006	0.971 ± 0.005	0.951 ± 0.007	0.935 ± 0.006	0.928 ± 0.011	0.921 ± 0.009	0.912 ± 0.013	0.904 ± 0.014	0.904 ± 0.013	0.906 ± 0.014	0.911 ± 0.013	0.911 ± 0.018	0.911 ± 0.021	0.911 ± 0.024	0.907 ± 0.024	0.899 ± 0.043
<i>Leptospermum</i> honey C (32% w/v)	1 ± 0	0.981 ± 0.006	0.976 ± 0.004	0.974 ± 0.007	0.975 ± 0.008	0.977 ± 0.007	0.980 ± 0.008	0.986 ± 0.008	0.988 ± 0.006	0.992 ± 0.009	0.996 ± 0.012	0.999 ± 0.019	1.010 ± 0.020	1.024 ± 0.023	1.037 ± 0.028	1.048 ± 0.029	1.039 ± 0.022
Garlic clove powder (75 mg/ml)	1 ± 0	0.982 ± 0.007	0.971 ± 0.016	0.964 ± 0.009	0.958 ± 0.019	0.977 ± 0.016	0.98 ± 0.013	1.011 ± 0.017	1.025 ± 0.022	1.028 ± 0.029	1.087 ± 0.007	1.128 ± 0.033	1.149 ± 0.029	1.189 ± 0.014	1.234 ± 0.017	1.297 ± 0.012	1.794 ± 0.094
Ginger rhizome powder (75 mg/ml)	1 ± 0	0.987 ± 0.013	0.984 ± 0.007	0.982 ± 0.004	0.983 ± 0.014	0.985 ± 0.006	0.989 ± 0.010	0.991 ± 0.010	0.991 ± 0.012	0.995 ± 0.025	1.009 ± 0.026	1.020 ± 0.035	1.028 ± 0.039	1.045 ± 0.031	1.051 ± 0.034	1.068 ± 0.024	1.493 ± 0.169
Cinnamon root powder (75 mg/ml)	1 ± 0	0.993 ± 0.008	0.988 ± 0.007	0.989 ± 0.005	0.998 ± 0.007	1.011 ± 0.011	1.013 ± 0.011	1.014 ± 0.016	1.021 ± 0.021	1.033 ± 0.026	1.049 ± 0.012	1.057 ± 0.021	1.061 ± 0.024	1.063 ± 0.026	1.070 ± 0.014	1.073 ± 0.024	1.305 ± 0.080
Turmeric root powder (75 mg/ml)	1 ± 0	0.962 ± 0.010	0.926 ± 0.007	0.900 ± 0.013	0.866 ± 0.014	0.961 ± 0.019	1.027 ± 0.030	1.041 ± 0.031	1.051 ± 0.027	1.078 ± 0.021	1.119 ± 0.011	1.132 ± 0.015	1.155 ± 0.029	1.160 ± 0.012	1.165 ± 0.012	1.177 ± 0.014	1.638 ± 0.104
Garlic tablet (75 mg/ml)	1 ± 0	0.988 ± 0.009	0.978 ± 0.008	0.970 ± 0.009	0.974 ± 0.010	0.986 ± 0.007	0.992 ± 0.010	0.996 ± 0.012	1.009 ± 0.014	1.011 ± 0.019	1.033 ± 0.021	1.041 ± 0.027	1.050 ± 0.019	1.066 ± 0.025	1.075 ± 0.026	1.105 ± 0.013	1.437 ± 0.138
Ginger tablet (75 mg/ml)	1 ± 0	0.981 ± 0.006	0.970 ± 0.007	0.963 ± 0.010	0.960 ± 0.013	0.982 ± 0.006	0.982 ± 0.007	0.987 ± 0.009	0.991 ± 0.007	1.003 ± 0.009	1.021 ± 0.015	1.035 ± 0.015	1.054 ± 0.017	1.061 ± 0.019	1.072 ± 0.017	1.078 ± 0.027	2.512 ± 0.641
Cinnamon tablet (75 mg/ml)	1 ± 0	0.981 ± 0.005	0.969 ± 0.011	0.958 ± 0.005	0.988 ± 0.011	0.993 ± 0.015	1.020 ± 0.013	1.023 ± 0.019	1.029 ± 0.023	1.038 ± 0.027	1.054 ± 0.031	1.072 ± 0.031	1.090 ± 0.012	1.095 ± 0.016	1.124 ± 0.012	1.132 ± 0.014	2.492 ± 0.386

Supplementary Table 1A (Cont.) The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain NCTC 13366.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Turmeric tablet (75 mg/ml)	1 ± 0	0.989 ± 0.014	0.981 ± 0.004	0.974 ± 0.009	0.980 ± 0.015	1.003 ± 0.023	1.006 ± 0.018	1.016 ± 0.026	1.017 ± 0.008	1.024 ± 0.017	1.052 ± 0.011	1.071 ± 0.012	1.095 ± 0.005	1.112 ± 0.006	1.119 ± 0.014	1.131 ± 0.009	1.751 ± 0.149
Artichoke (75 mg/ml)	1 ± 0	0.979 ± 0.013	0.973 ± 0.006	0.971 ± 0.010	0.971 ± 0.009	0.968 ± 0.011	0.968 ± 0.012	0.967 ± 0.013	0.966 ± 0.015	0.958 ± 0.014	0.957 ± 0.014	0.959 ± 0.013	0.958 ± 0.013	0.961 ± 0.011	0.962 ± 0.011	0.964 ± 0.011	0.961 ± 0.015
Aloe vera gel (32% w/v)	1 ± 0	0.986 ± 0.008	0.969 ± 0.009	0.963 ± 0.004	0.967 ± 0.013	0.983 ± 0.013	1.002 ± 0.020	1.020 ± 0.032	1.035 ± 0.015	1.060 ± 0.019	1.068 ± 0.018	1.104 ± 0.013	1.111 ± 0.014	1.139 ± 0.015	1.152 ± 0.011	1.176 ± 0.007	1.404 ± 0.049
Coconut oil (32% v/v)	1 ± 0	0.984 ± 0.009	0.966 ± 0.010	0.949 ± 0.011	0.960 ± 0.014	0.982 ± 0.008	0.992 ± 0.008	0.994 ± 0.010	0.993 ± 0.018	0.995 ± 0.019	1.015 ± 0.015	1.024 ± 0.017	1.028 ± 0.012	1.040 ± 0.023	1.047 ± 0.015	1.054 ± 0.013	2.51 ± 0.358
Peppermint oil (32% v/v)	1 ± 0	0.942 ± 0.036	0.839 ± 0.029	0.775 ± 0.013	0.706 ± 0.026	0.679 ± 0.033	0.678 ± 0.034	0.653 ± 0.047	0.590 ± 0.061	0.635 ± 0.081	0.733 ± 0.040	0.822 ± 0.051	0.946 ± 0.029	1.189 ± 0.012	1.234 ± 0.035	1.297 ± 0.021	1.506 ± 0.094
Allicin (75 mg/ml)	1 ± 0	0.990 ± 0.009	0.985 ± 0.006	0.985 ± 0.009	1.011 ± 0.015	1.017 ± 0.020	1.023 ± 0.030	1.034 ± 0.019	1.038 ± 0.026	1.046 ± 0.025	1.061 ± 0.012	1.075 ± 0.008	1.079 ± 0.011	1.084 ± 0.012	1.087 ± 0.014	1.090 ± 0.005	1.116 ± 0.042
trans-Cinnamaldehyde (32 v/v)	1 ± 0	0.983 ± 0.029	0.960 ± 0.008	0.945 ± 0.014	0.915 ± 0.016	0.916 ± 0.018	0.919 ± 0.020	0.924 ± 0.022	0.928 ± 0.006	0.931 ± 0.008	0.986 ± 0.013	0.996 ± 0.024	1.005 ± 0.007	1.089 ± 0.012	1.157 ± 0.036	1.178 ± 0.013	1.389 ± 0.026
Menthol (75 mg/ml)	1 ± 0	0.977 ± 0.006	0.972 ± 0.008	0.964 ± 0.006	0.960 ± 0.005	0.972 ± 0.007	0.976 ± 0.008	0.982 ± 0.011	0.995 ± 0.014	0.996 ± 0.005	1.007 ± 0.010	1.018 ± 0.008	1.032 ± 0.010	1.049 ± 0.009	1.057 ± 0.009	1.0631 ± 0.022	1.396 ± 0.171
Zingerone (75 mg/ml)	1 ± 0	0.986 ± 0.008	0.942 ± 0.006	0.914 ± 0.012	0.887 ± 0.020	0.870 ± 0.010	0.865 ± 0.012	0.830 ± 0.030	0.7971 ± 0.017	0.782 ± 0.049	0.999 ± 0.042	1.159 ± 0.055	1.349 ± 0.038	1.559 ± 0.024	1.969 ± 0.054	2.050 ± 0.022	2.258 ± 0.067
SDW	1 ± 0	0.964 ± 0.011	0.956 ± 0.009	0.948 ± 0.014	0.961 ± 0.018	0.963 ± 0.013	0.969 ± 0.013	0.970 ± 0.016	0.975 ± 0.015	0.979 ± 0.018	0.982 ± 0.024	0.999 ± 0.031	1.015 ± 0.024	1.032 ± 0.022	1.048 ± 0.019	1.083 ± 0.024	3.906 ± 0.474
DMSO (2.5% v/v)	1 ± 0	0.970 ± 0.013	0.963 ± 0.008	0.956 ± 0.012	0.950 ± 0.029	0.953 ± 0.031	0.953 ± 0.029	0.955 ± 0.029	0.958 ± 0.027	0.960 ± 0.028	0.975 ± 0.032	0.989 ± 0.033	1.000 ± 0.035	1.006 ± 0.035	1.022 ± 0.039	1.058 ± 0.040	4.072 ± 0.573
Fidaxomicin (16 mg/ml)	1 ± 0	0.972 ± 0.011	0.958 ± 0.020	0.955 ± 0.019	0.956 ± 0.013	0.959 ± 0.017	0.959 ± 0.019	0.959 ± 0.016	0.962 ± 0.015	0.962 ± 0.013	0.965 ± 0.017	0.961 ± 0.025	0.961 ± 0.025	0.961 ± 0.008	0.960 ± 0.019	0.960 ± 0.024	0.944 ± 0.023
Vancomycin (16 mg/ml)	1 ± 0	0.981 ± 0.008	0.973 ± 0.006	0.969 ± 0.004	0.960 ± 0.009	0.964 ± 0.013	0.964 ± 0.011	0.962 ± 0.007	0.964 ± 0.008	0.967 ± 0.011	0.972 ± 0.010	0.975 ± 0.006	0.976 ± 0.013	0.977 ± 0.007	0.978 ± 0.010	0.974 ± 0.015	0.967 ± 0.025

OD, optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour

Supplementary Table 1B The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain NCTC 13366.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh onion bulb (25% v/v)	1 ± 0	0.974 ± 0.010	0.955 ± 0.009	0.942 ± 0.015	0.944 ± 0.007	0.955 ± 0.009	0.959 ± 0.008	0.969 ± 0.009	0.979 ± 0.025	1.004 ± 0.011	1.026 ± 0.031	1.039 ± 0.011	1.052 ± 0.013	1.063 ± 0.007	1.076 ± 0.020	1.091 ± 0.017	1.144 ± 0.039
Fresh onion bulb (12.5% v/v)	1 ± 0	0.966 ± 0.017	0.929 ± 0.012	0.918 ± 0.008	0.918 ± 0.004	0.914 ± 0.012	0.916 ± 0.007	0.0916 ± 0.015	0.921 ± 0.013	0.922 ± 0.015	0.952 ± 0.019	0.970 ± 0.008	0.980 ± 0.008	0.995 ± 0.005	1.003 ± 0.012	1.016 ± 0.006	1.805 ± 0.386
Artichoke (37.5 mg/ml)	1 ± 0	0.996 ± 0.004	0.970 ± 0.008	0.955 ± 0.005	0.944 ± 0.010	0.943 ± 0.012	0.949 ± 0.014	0.948 ± 0.014	0.948 ± 0.009	0.951 ± 0.006	0.0959 ± 0.006	0.958 ± 0.008	0.959 ± 0.009	0.955 ± 0.007	0.955 ± 0.011	0.952 ± 0.012	0.916 ± 0.062
Artichoke (18.8 mg/ml)	1 ± 0	0.991 ± 0.006	0.968 ± 0.007	0.957 ± 0.008	0.953 ± 0.007	0.972 ± 0.005	1.003 ± 0.019	1.007 ± 0.015	1.005 ± 0.006	1.015 ± 0.013	1.026 ± 0.017	1.026 ± 0.007	1.029 ± 0.008	1.031 ± 0.008	1.030 ± 0.005	1.026 ± 0.028	1.002 ± 0.093
Leptospermum honey A (8% w/v)	1 ± 0	0.986 ± 0.007	0.979 ± 0.006	0.964 ± 0.006	0.949 ± 0.006	0.940 ± 0.007	0.933 ± 0.007	0.932 ± 0.007	0.924 ± 0.013	0.0912 ± 0.022	0.901 ± 0.008	0.893 ± 0.009	0.897 ± 0.012	0.900 ± 0.016	0.896 ± 0.013	0.903 ± 0.020	0.866 ± 0.033
Leptospermum honey A (4% w/v)	1 ± 0	0.983 ± 0.007	0.976 ± 0.009	0.968 ± 0.006	0.961 ± 0.007	0.0959 ± 0.011	0.959 ± 0.010	0.954 ± 0.006	0.946 ± 0.010	0.034 ± 0.008	0.941 ± 0.006	0.946 ± 0.007	0.949 ± 0.005	0.950 ± 0.008	0.946 ± 0.006	0.936 ± 0.016	0.946 ± 0.036
Leptospermum honey B (8% w/v)	1 ± 0	0.992 ± 0.004	0.980 ± 0.004	0.968 ± 0.004	0.953 ± 0.004	0.948 ± 0.006	0.940 ± 0.005	0.935 ± 0.004	0.921 ± 0.012	0.912 ± 0.009	0.915 ± 0.025	0.916 ± 0.013	0.932 ± 0.008	0.929 ± 0.005	0.925 ± 0.007	0.925 ± 0.013	0.884 ± 0.10
Leptospermum honey B (4% w/v)	1 ± 0	0.988 ± 0.009	0.982 ± 0.005	0.979 ± 0.004	0.974 ± 0.005	0.972 ± 0.004	0.970 ± 0.004	0.961 ± 0.005	0.956 ± 0.006	0.956 ± 0.015	0.956 ± 0.019	0.958 ± 0.013	0.960 ± 0.012	0.963 ± 0.006	0.971 ± 0.011	0.964 ± 0.012	0.936 ± 0.062
Leptospermum honey C (8% w/v)	1 ± 0	0.985 ± 0.006	0.977 ± 0.008	0.963 ± 0.004	0.948 ± 0.005	0.941 ± 0.004	0.936 ± 0.004	0.922 ± 0.008	0.913 ± 0.008	0.911 ± 0.012	0.912 ± 0.015	0.914 ± 0.013	0.915 ± 0.014	0.919 ± 0.017	0.920 ± 0.006	0.926 ± 0.007	0.891 ± 0.029
Leptospermum honey C (4% w/v)	1 ± 0	0.991 ± 0.004	0.978 ± 0.008	0.972 ± 0.008	0.970 ± 0.008	0.966 ± 0.004	0.962 ± 0.009	0.955 ± 0.014	0.951 ± 0.018	0.953 ± 0.018	0.961 ± 0.014	0.966 ± 0.012	0.969 ± 0.007	0.977 ± 0.006	0.980 ± 0.005	0.983 ± 0.007	0.978 ± 0.048
Allicin (37.5 mg/ml)	1 ± 0	0.988 ± 0.004	0.966 ± 0.013	0.962 ± 0.005	0.959 ± 0.007	0.956 ± 0.009	0.955 ± 0.011	0.945 ± 0.007	0.934 ± 0.008	0.930 ± 0.008	0.931 ± 0.006	0.933 ± 0.007	0.939 ± 0.011	0.944 ± 0.006	0.947 ± 0.005	0.953 ± 0.014	1.446 ± 0.250
Allicin (18.8 mg/ml)	1 ± 0	0.978 ± 0.006	0.962 ± 0.005	0.956 ± 0.007	0.965 ± 0.009	0.952 ± 0.007	0.970 ± 0.009	0.975 ± 0.006	0.975 ± 0.008	0.975 ± 0.006	0.981 ± 0.007	0.997 ± 0.008	1.026 ± 0.012	1.054 ± 0.022	1.076 ± 0.006	1.090 ± 0.015	1.681 ± 0.395
SDW	1 ± 0	0.964 ± 0.011	0.956 ± 0.009	0.948 ± 0.014	0.961 ± 0.018	0.963 ± 0.013	0.969 ± 0.013	0.970 ± 0.016	0.975 ± 0.015	0.979 ± 0.018	0.982 ± 0.024	0.999 ± 0.031	1.015 ± 0.024	1.032 ± 0.022	1.048 ± 0.019	1.083 ± 0.024	3.906 ± 0.474
DMSO (2.5% v/v)	1 ± 0	0.964 ± 0.011	0.956 ± 0.009	0.948 ± 0.014	0.961 ± 0.018	0.963 ± 0.013	0.969 ± 0.013	0.970 ± 0.016	0.975 ± 0.015	0.979 ± 0.018	0.982 ± 0.024	0.999 ± 0.031	1.015 ± 0.024	1.032 ± 0.022	1.048 ± 0.019	1.083 ± 0.024	3.906 ± 0.474
Fidaxomicin (8 mg/ml)	1 ± 0	0.974 ± 0.007	0.937 ± 0.005	0.926 ± 0.008	0.908 ± 0.008	0.908 ± 0.007	0.908 ± 0.007	0.914 ± 0.009	0.914 ± 0.012	0.943 ± 0.0012	0.951 ± 0.005	0.955 ± 0.010	0.958 ± 0.006	0.958 ± 0.006	0.954 ± 0.019	0.954 ± 0.009	0.922 ± 0.052
Fidaxomicin (4 mg/ml)	1 ± 0	0.975 ± 0.006	0.959 ± 0.006	0.953 ± 0.006	0.953 ± 0.006	0.956 ± 0.007	0.966 ± 0.008	0.972 ± 0.012	0.978 ± 0.006	0.978 ± 0.006	0.981 ± 0.009	0.994 ± 0.009	1.020 ± 0.015	1.020 ± 0.018	1.025 ± 0.005	1.017 ± 0.016	1.006 ± 0.033

OD, optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour.

Supplementary Table 2A The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain ATTC 700057.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh garlic bulb (50% v/v)	1 ± 0	0.971 ± 0.015	0.959 ± 0.007	0.939 ± 0.009	0.936 ± 0.007	0.923 ± 0.009	0.918 ± 0.008	0.906 ± 0.008	0.903 ± 0.009	0.933 ± 0.025	0.968 ± 0.018	0.988 ± 0.015	1.018 ± 0.010	1.026 ± 0.017	1.063 ± 0.016	1.074 ± 0.008	1.234 ± 0.014
Fresh ginger rhizome (50% v/v)	1 ± 0	0.988 ± 0.006	0.972 ± 0.009	0.955 ± 0.008	0.948 ± 0.007	0.971 ± 0.018	0.987 ± 0.019	1.021 ± 0.012	1.026 ± 0.023	1.063 ± 0.021	1.090 ± 0.022	1.113 ± 0.020	1.131 ± 0.015	1.147 ± 0.011	1.152 ± 0.010	1.173 ± 0.025	1.316 ± 0.041
Fresh onion bulb (50% v/v)	1 ± 0	0.988 ± 0.012	0.976 ± 0.009	0.974 ± 0.004	0.999 ± 0.015	1.005 ± 0.016	1.010 ± 0.017	1.017 ± 0.020	0.987 ± 0.016	0.980 ± 0.010	1.013 ± 0.020	1.027 ± 0.008	1.037 ± 0.017	1.036 ± 0.016	1.036 ± 0.007	1.041 ± 0.020	1.014 ± 0.033
<i>Leptospermum</i> honey A (32% w/v)	1 ± 0	0.982 ± 0.005	0.978 ± 0.004	0.974 ± 0.004	0.983 ± 0.005	0.986 ± 0.004	0.988 ± 0.007	0.990 ± 0.005	0.996 ± 0.016	1.000 ± 0.007	1.024 ± 0.007	1.018 ± 0.006	1.004 ± 0.013	1.004 ± 0.009	0.985 ± 0.014	0.970 ± 0.019	0.950 ± 0.042
<i>Leptospermum</i> honey B (32% w/v)	1 ± 0	0.984 ± 0.005	0.979 ± 0.007	0.974 ± 0.008	0.979 ± 0.015	0.985 ± 0.008	0.989 ± 0.008	0.994 ± 0.009	0.998 ± 0.007	1.002 ± 0.008	1.022 ± 0.012	1.013 ± 0.007	0.995 ± 0.008	0.987 ± 0.010	0.980 ± 0.010	0.965 ± 0.011	0.949 ± 0.034
<i>Leptospermum</i> honey C (32% w/v)	1 ± 0	0.980 ± 0.006	0.973 ± 0.006	0.968 ± 0.008	0.971 ± 0.007	0.973 ± 0.006	0.977 ± 0.007	0.980 ± 0.008	0.984 ± 0.010	0.990 ± 0.012	0.992 ± 0.023	1.002 ± 0.020	1.023 ± 0.013	1.036 ± 0.015	1.049 ± 0.015	1.058 ± 0.014	1.053 ± 0.075
Garlic clove powder (75 mg/ml)	1 ± 0	0.981 ± 0.005	0.977 ± 0.006	0.8791 ± 0.009	0.962 ± 0.030	0.991 ± 0.005	0.996 ± 0.025	1.005 ± 0.027	1.012 ± 0.030	1.018 ± 0.014	1.059 ± 0.020	1.112 ± 0.010	1.166 ± 0.011	1.210 ± 0.015	1.264 ± 0.011	1.287 ± 0.015	1.882 ± 0.041
Ginger rhizome powder (75 mg/ml)	1 ± 0	0.985 ± 0.006	0.977 ± 0.007	0.973 ± 0.006	0.972 ± 0.010	0.988 ± 0.009	0.998 ± 0.010	0.999 ± 0.010	1.001 ± 0.011	1.003 ± 0.012	1.008 ± 0.006	1.018 ± 0.015	1.026 ± 0.007	1.034 ± 0.010	1.045 ± 0.008	1.054 ± 0.011	1.466 ± 0.030
Cinnamon root powder (75 mg/ml)	1 ± 0	0.992 ± 0.004	0.989 ± 0.003	0.978 ± 0.009	1.005 ± 0.009	1.021 ± 0.010	1.026 ± 0.011	1.028 ± 0.012	1.030 ± 0.014	1.030 ± 0.007	1.034 ± 0.010	1.040 ± 0.006	1.044 ± 0.007	1.049 ± 0.006	1.055 ± 0.010	1.062 ± 0.009	1.225 ± 0.020
Turmeric root powder (75 mg/ml)	1 ± 0	0.942 ± 0.011	0.890 ± 0.011	0.851 ± 0.014	0.814 ± 0.013	0.742 ± 0.011	0.870 ± 0.051	0.921 ± 0.020	1.003 ± 0.035	1.020 ± 0.023	1.051 ± 0.006	1.084 ± 0.013	1.112 ± 0.011	1.133 ± 0.015	1.147 ± 0.016	1.174 ± 0.010	2.147 ± 0.107
Garlic tablet (75 mg/ml)	1 ± 0	0.984 ± 0.013	0.972 ± 0.008	0.962 ± 0.010	0.986 ± 0.010	1.003 ± 0.007	1.010 ± 0.009	1.015 ± 0.012	1.017 ± 0.007	1.023 ± 0.008	1.049 ± 0.011	1.064 ± 0.014	1.077 ± 0.006	1.091 ± 0.025	1.112 ± 0.007	1.134 ± 0.009	1.652 ± 0.142
Ginger tablet (75 mg/ml)	1 ± 0	0.976 ± 0.008	0.965 ± 0.006	0.961 ± 0.009	0.958 ± 0.014	0.958 ± 0.009	0.977 ± 0.015	0.986 ± 0.006	1.003 ± 0.021	1.010 ± 0.010	1.031 ± 0.013	1.049 ± 0.015	1.087 ± 0.009	1.094 ± 0.019	1.111 ± 0.008	1.125 ± 0.008	3.267 ± 0.582
Cinnamon tablet (75 mg/ml)	1 ± 0	0.989 ± 0.013	0.984 ± 0.009	0.962 ± 0.007	0.959 ± 0.009	0.984 ± 0.008	1.007 ± 0.013	1.013 ± 0.013	1.024 ± 0.007	1.032 ± 0.009	1.056 ± 0.012	1.073 ± 0.007	1.086 ± 0.009	1.100 ± 0.011	1.115 ± 0.010	1.125 ± 0.009	2.320 ± 0.028

Supplementary 2A (Cont.) The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain ATTC 700057.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Turmeric tablet (75 mg/ml)	1 ± 0	0.989 ± 0.011	0.985 ± 0.009	0.970 ± 0.009	0.982 ± 0.017	0.975 ± 0.011	0.993 ± 0.012	1.011 ± 0.009	1.015 ± 0.009	1.022 ± 0.006	1.085 ± 0.006	1.133 ± 0.009	1.194 ± 0.012	1.246 ± 0.015	1.270 ± 0.011	1.293 ± 0.020	1.733 ± 0.161
Artichoke (75 mg/ml)	1 ± 0	0.985 ± 0.008	0.980 ± 0.006	0.980 ± 0.007	0.976 ± 0.006	0.976 ± 0.008	0.974 ± 0.007	0.972 ± 0.007	0.975 ± 0.007	0.964 ± 0.006	0.959 ± 0.010	0.963 ± 0.008	0.966 ± 0.011	0.968 ± 0.012	0.971 ± 0.010	0.972 ± 0.008	0.969 ± 0.023
Aloe vera gel (32% w/v)	1 ± 0	0.980 ± 0.014	0.977 ± 0.008	0.975 ± 0.008	0.972 ± 0.010	0.969 ± 0.006	0.976 ± 0.011	0.974 ± 0.009	0.977 ± 0.015	0.983 ± 0.009	1.017 ± 0.012	1.022 ± 0.007	1.025 ± 0.010	1.042 ± 0.008	1.044 ± 0.006	1.051 ± 0.013	1.734 ± 0.049
Coconut oil (32% v/v)	1 ± 0	0.977 ± 0.006	0.973 ± 0.018	0.947 ± 0.014	0.919 ± 0.023	0.870 ± 0.011	0.865 ± 0.011	0.974 ± 0.046	1.000 ± 0.016	1.021 ± 0.020	1.069 ± 0.009	1.079 ± 0.009	1.168 ± 0.050	1.193 ± 0.006	1.2295 ± 0.022	1.281 ± 0.033	2.331 ± 0.372
Peppermint oil (32% v/v)	1 ± 0	0.959 ± 0.014	0.852 ± 0.012	0.821 ± 0.009	0.798 ± 0.015	0.727 ± 0.008	0.713 ± 0.020	0.671 ± 0.010	0.655 ± 0.011	0.679 ± 0.025	0.726 ± 0.021	0.743 ± 0.013	0.756 ± 0.010	0.812 ± 0.036	0.848 ± 0.025	0.868 ± 0.017	1.183 ± 0.128
Allicin (75 mg/ml)	1 ± 0	0.992 ± 0.007	0.988 ± 0.005	0.986 ± 0.011	1.003 ± 0.005	1.011 ± 0.004	1.020 ± 0.008	1.032 ± 0.010	1.037 ± 0.006	1.044 ± 0.005	1.060 ± 0.008	1.072 ± 0.006	1.074 ± 0.006	1.078 ± 0.008	1.080 ± 0.007	1.081 ± 0.008	1.080 ± 0.037
trans-Cinnamaldehyde (32% v/v)	1 ± 0	0.959 ± 0.014	0.921 ± 0.021	0.896 ± 0.016	0.853 ± 0.020	0.890 ± 0.025	0.914 ± 0.015	0.915 ± 0.006	0.923 ± 0.007	0.919 ± 0.016	0.983 ± 0.013	0.996 ± 0.014	1.017 ± 0.008	1.041 ± 0.010	1.061 ± 0.014	1.089 ± 0.017	1.463 ± 0.203
Menthol (75 mg/ml)	1 ± 0	0.979 ± 0.015	0.966 ± 0.018	0.963 ± 0.013	0.959 ± 0.009	0.929 ± 0.010	0.925 ± 0.014	0.952 ± 0.010	0.975 ± 0.011	0.979 ± 0.017	0.995 ± 0.030	0.999 ± 0.013	1.006 ± 0.013	1.032 ± 0.009	1.063 ± 0.015	1.093 ± 0.014	1.443 ± 0.183
Zingerone (75 mg/ml)	1 ± 0	0.974 ± 0.012	0.922 ± 0.011	0.862 ± 0.014	0.840 ± 0.013	0.815 ± 0.026	0.792 ± 0.016	0.764 ± 0.034	0.708 ± 0.030	0.670 ± 0.014	0.788 ± 0.015	1.037 ± 0.067	1.188 ± 0.045	1.223 ± 0.042	1.467 ± 0.101	1.712 ± 0.108	2.065 ± 0.105
SDW	1 ± 0	0.973 ± 0.006	0.964 ± 0.011	0.958 ± 0.008	0.960 ± 0.005	0.963 ± 0.007	0.964 ± 0.010	0.964 ± 0.010	0.973 ± 0.19	0.978 ± 0.022	0.999 ± 0.026	1.015 ± 0.019	1.036 ± 0.012	1.060 ± 0.029	1.099 ± 0.019	1.157 ± 0.104	3.428 ± 0.488
DMSO (2.5% v/v)	1 ± 0	0.982 ± 0.004	0.970 ± 0.009	0.956 ± 0.004	0.952 ± 0.0001	0.962 ± 0.011	0.977 ± 0.009	0.979 ± 0.011	0.982 ± 0.004	0.989 ± 0.004	1.010 ± 0.015	1.026 ± 0.014	1.041 ± 0.009	1.053 ± 0.006	1.099 ± 0.017	1.140 ± 0.013	3.027 ± 0.120
Fidaxomicin (16 mg/ml)	1 ± 0	0.971 ± 0.012	0.964 ± 0.007	0.957 ± 0.011	0.954 ± 0.007	0.956 ± 0.010	0.958 ± 0.008	0.962 ± 0.007	0.960 ± 0.010	0.963 ± 0.013	0.968 ± 0.006	0.964 ± 0.012	0.963 ± 0.014	0.961 ± 0.016	0.957 ± 0.013	0.951 ± 0.011	0.937 ± 0.013
Vancomycin (16 mg/ml)	1 ± 0	0.972 ± 0.010	0.960 ± 0.013	0.960 ± 0.006	0.965 ± 0.006	0.967 ± 0.007	0.972 ± 0.011	0.979 ± 0.011	0.981 ± 0.013	0.983 ± 0.006	0.993 ± 0.011	1.001 ± 0.013	1.004 ± 0.008	1.000 ± 0.011	1.001 ± 0.008	0.999 ± 0.013	0.991 ± 0.032

OD, Optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour.

Supplementary Table 2B The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain ATTC 700057.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh onion bulb (25% v/v)	1 ± 0	0.970 ± 0.009	0.965 ± 0.005	0.965 ± 0.007	0.963 ± 0.007	0.967 ± 0.012	0.97 ± 0.012	0.982 ± 0.013	0.991 ± 0.009	1.030 ± 0.009	1.054 ± 0.005	1.074 ± 0.008	1.086 ± 0.009	1.098 ± 0.012	1.098 ± 0.012	1.114 ± 0.011	1.224 ± 0.125
Fresh onion bulb (12.5% v/v)	1 ± 0	0.979 ± 0.005	0.950 ± 0.005	0.948 ± 0.009	0.938 ± 0.010	0.950 ± 0.005	0.950 ± 0.012	0.953 ± 0.008	0.957 ± 0.009	0.960 ± 0.005	0.990 ± 0.008	1.012 ± 0.005	1.024 ± 0.006	1.033 ± 0.010	1.045 ± 0.010	1.059 ± 0.006	1.851 ± 0.216
Artichoke (37.5 mg/ml)	1 ± 0	0.984 ± 0.007	0.979 ± 0.006	0.962 ± 0.008	0.974 ± 0.013	0.985 ± 0.010	0.988 ± 0.0098	0.991 ± 0.011	0.993 ± 0.012	1.000 ± 0.008	1.008 ± 0.006	1.011 ± 0.016	1.015 ± 0.007	1.017 ± 0.006	1.017 ± 0.014	1.016 ± 0.016	0.985 ± 0.027
Artichoke (18.8 mg/ml)	1 ± 0	0.993 ± 0.005	0.979 ± 0.010	0.977 ± 0.007	0.977 ± 0.008	0.982 ± 0.008	0.983 ± 0.009	0.985 ± 0.013	0.987 ± 0.011	0.989 ± 0.12	0.993 ± 0.11	0.997 ± 0.007	0.998 ± 0.008	1.001 ± 0.013	1.000 ± 0.014	1.000 ± 0.014	1.002 ± 0.028
Leptospermum honey A (8% w/v)	1 ± 0	0.979 ± 0.005	0.976 ± 0.006	0.971 ± 0.009	0.971 ± 0.006	0.962 ± 0.005	0.954 ± 0.006	0.940 ± 0.005	0.929 ± 0.12	0.933 ± 0.006	0.936 ± 0.008	0.940 ± 0.007	0.947 ± 0.006	0.949 ± 0.006	0.951 ± 0.008	0.951 ± 0.009	0.927 ± 0.031
Leptospermum honey A (4% w/v)	1 ± 0	0.987 ± 0.005	0.975 ± 0.005	0.967 ± 0.008	0.963 ± 0.011	0.967 ± 0.005	0.964 ± 0.006	0.956 ± 0.005	0.946 ± 0.005	0.938 ± 0.009	0.941 ± 0.007	0.946 ± 0.007	0.957 ± 0.008	0.960 ± 0.006	0.967 ± 0.010	0.973 ± 0.006	0.963 ± 0.032
Leptospermum honey B (8% w/v)	1 ± 0	0.965 ± 0.018	0.957 ± 0.006	0.952 ± 0.009	0.948 ± 0.008	0.947 ± 0.014	0.944 ± 0.012	0.933 ± 0.011	0.940 ± 0.009	0.940 ± 0.011	0.946 ± 0.007	0.948 ± 0.016	0.934 ± 0.008	0.933 ± 0.008	0.932 ± 0.008	0.963 ± 0.019	0.919 ± 0.046
Leptospermum honey B (4% w/v)	1 ± 0	0.983 ± 0.006	0.977 ± 0.004	0.972 ± 0.006	0.973 ± 0.007	0.969 ± 0.006	0.967 ± 0.008	0.954 ± 0.008	0.946 ± 0.007	0.946 ± 0.008	0.956 ± 0.006	0.961 ± 0.005	0.962 ± 0.004	0.964 ± 0.005	0.964 ± 0.004	0.967 ± 0.005	0.948 ± 0.020
Leptospermum honey C (8% w/v)	1 ± 0	0.987 ± 0.010	0.979 ± 0.007	0.974 ± 0.009	0.973 ± 0.008	0.968 ± 0.007	0.964 ± 0.010	0.951 ± 0.015	0.926 ± 0.020	0.929 ± 0.015	0.932 ± 0.017	0.936 ± 0.007	0.940 ± 0.010	0.943 ± 0.011	0.948 ± 0.009	0.925 ± 0.015	0.925 ± 0.015
Leptospermum honey C (4% w/v)	1 ± 0	0.986 ± 0.007	0.974 ± 0.011	0.971 ± 0.005	0.971 ± 0.008	0.967 ± 0.008	0.967 ± 0.007	0.956 ± 0.009	0.949 ± 0.010	0.927 ± 0.013	0.925 ± 0.012	0.930 ± 0.008	0.940 ± 0.013	0.942 ± 0.005	0.943 ± 0.008	0.944 ± 0.008	0.986 ± 0.059
Allicin (37.5 mg/ml)	1 ± 0	0.974 ± 0.010	0.933 ± 0.007	0.922 ± 0.008	0.919 ± 0.011	0.918 ± 0.011	0.913 ± 0.011	0.913 ± 0.007	0.908 ± 0.013	0.908 ± 0.012	0.913 ± 0.007	0.916 ± 0.009	0.920 ± 0.007	0.923 ± 0.008	0.932 ± 0.008	0.935 ± 0.008	1.796 ± 0.207
Allicin (18.8 mg/ml)	1 ± 0	0.977 ± 0.015	0.968 ± 0.008	0.966 ± 0.008	0.964 ± 0.014	0.953 ± 0.010	0.950 ± 0.009	0.951 ± 0.014	0.949 ± 0.010	0.944 ± 0.011	0.951 ± 0.012	0.953 ± 0.006	0.959 ± 0.014	0.966 ± 0.011	0.983 ± 0.014	1.795 ± 0.159	1.795 ± 0.159
SDW	1 ± 0	0.973 ± 0.006	0.964 ± 0.011	0.958 ± 0.008	0.960 ± 0.005	0.963 ± 0.007	0.964 ± 0.010	0.964 ± 0.010	0.973 ± 0.19	0.978 ± 0.022	0.999 ± 0.026	1.015 ± 0.019	1.036 ± 0.012	1.060 ± 0.029	1.099 ± 0.019	1.157 ± 0.104	3.428 ± 0.488
DMSO (2.5% v/v)	1 ± 0	0.982 ± 0.004	0.970 ± 0.009	0.956 ± 0.004	0.952 ± 0.0001	0.962 ± 0.011	0.977 ± 0.009	0.979 ± 0.011	0.982 ± 0.004	0.989 ± 0.004	1.010 ± 0.015	1.026 ± 0.014	1.041 ± 0.009	1.053 ± 0.006	1.099 ± 0.017	1.140 ± 0.013	3.027 ± 0.120
Fidaxomicin (8 mg/ml)	1 ± 0	0.964 ± 0.010	0.953 ± 0.005	0.950 ± 0.005	0.950 ± 0.006	0.947 ± 0.006	0.947 ± 0.011	0.953 ± 0.009	0.956 ± 0.006	0.956 ± 0.006	0.968 ± 0.006	0.977 ± 0.009	0.974 ± 0.014	0.977 ± 0.006	0.980 ± 0.008	0.979 ± 0.017	0.950 ± 0.026
Fidaxomicin (4 mg/ml)	1 ± 0	0.963 ± 0.021	0.945 ± 0.009	0.942 ± 0.006	0.942 ± 0.010	0.945 ± 0.009	0.957 ± 0.009	0.957 ± 0.009	0.960 ± 0.009	0.963 ± 0.007	0.963 ± 0.007	0.965 ± 0.007	0.965 ± 0.015	0.965 ± 0.008	0.962 ± 0.006	0.971 ± 0.009	0.942 ± 0.020

OD, optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour.

Supplementary Table 3A The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain R11446.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh garlic bulb (50% v/v)	1 ± 0	0.976 ± 0.016	0.968 ± 0.006	0.928 ± 0.010	0.923 ± 0.006	0.941 ± 0.012	0.951 ± 0.008	0.959 ± 0.006	0.970 ± 0.008	0.985 ± 0.011	1.010 ± 0.009	1.014 ± 0.012	1.029 ± 0.012	1.053 ± 0.008	1.065 ± 0.014	1.069 ± 0.012	1.192 ± 0.038
Fresh ginger rhizome (50% v/v)	1 ± 0	0.989 ± 0.007	0.978 ± 0.005	0.971 ± 0.007	0.968 ± 0.017	0.983 ± 0.007	0.988 ± 0.009	1.005 ± 0.014	1.012 ± 0.013	1.017 ± 0.007	1.062 ± 0.020	1.072 ± 0.016	1.115 ± 0.007	1.119 ± 0.013	1.126 ± 0.009	1.137 ± 0.016	1.353 ± 0.059
Fresh onion bulb (50% v/v)	1 ± 0	0.976 ± 0.006	0.974 ± 0.006	0.960 ± 0.015	0.980 ± 0.008	0.986 ± 0.009	0.992 ± 0.013	0.995 ± 0.006	1.002 ± 0.007	1.005 ± 0.012	0.989 ± 0.014	0.992 ± 0.017	0.995 ± 0.012	1.010 ± 0.010	1.009 ± 0.012	1.012 ± 0.010	1.022 ± 0.016
<i>Leptospermum</i> honey A (32% w/v)	1 ± 0	0.986 ± 0.006	0.980 ± 0.006	0.978 ± 0.006	0.980 ± 0.007	0.981 ± 0.006	0.985 ± 0.009	0.989 ± 0.012	0.993 ± 0.010	0.991 ± 0.004	1.016 ± 0.010	1.023 ± 0.015	1.021 ± 0.014	1.000 ± 0.010	0.995 ± 0.005	0.990 ± 0.013	0.969 ± 0.012
<i>Leptospermum</i> honey B (32% w/v)	1 ± 0	0.983 ± 0.008	0.978 ± 0.009	0.977 ± 0.009	0.972 ± 0.009	0.972 ± 0.009	0.982 ± 0.015	0.988 ± 0.013	0.991 ± 0.013	0.994 ± 0.007	1.022 ± 0.014	1.012 ± 0.017	0.997 ± 0.012	0.982 ± 0.017	± 0.017	0.968 ± 0.015	0.901 ± 0.053
<i>Leptospermum</i> honey C (32% w/v)	1 ± 0	0.968 ± 0.014	0.954 ± 0.005	0.951 ± 0.008	0.948 ± 0.011	0.958 ± 0.012	0.965 ± 0.010	0.967 ± 0.008	0.981 ± 0.010	0.990 ± 0.006	1.003 ± 0.014	1.020 ± 0.009	0.989 ± 0.018	0.990 ± 0.011	1.025 ± 0.012	1.016 ± 0.015	1.012 ± 0.039
Garlic clove powder (75 mg/ml)	1 ± 0	0.984 ± 0.005	0.974 ± 0.005	0.969 ± 0.012	0.958 ± 0.006	0.991 ± 0.012	1.004 ± 0.008	1.011 ± 0.016	1.022 ± 0.006	1.028 ± 0.006	1.075 ± 0.014	1.132 ± 0.014	1.182 ± 0.010	1.248 ± 0.027	1.327 ± 0.020	1.368 ± 0.011	1.845 ± 0.057
Ginger rhizome powder (75 mg/ml)	1 ± 0	0.987 ± 0.005	0.978 ± 0.004	0.972 ± 0.006	0.980 ± 0.005	0.986 ± 0.005	0.989 ± 0.008	1.004 ± 0.016	1.007 ± 0.009	1.017 ± 0.014	1.032 ± 0.010	1.045 ± 0.012	1.059 ± 0.010	1.069 ± 0.007	1.085 ± 0.015	1.096 ± 0.012	1.364 ± 0.080
Cinnamon root powder (75 mg/ml)	1 ± 0	0.994 ± 0.005	0.988 ± 0.005	0.979 ± 0.003	0.989 ± 0.014	1.006 ± 0.012	1.014 ± 0.006	1.028 ± 0.009	1.028 ± 0.008	1.037 ± 0.012	1.052 ± 0.013	1.063 ± 0.010	1.070 ± 0.012	1.073 ± 0.017	1.079 ± 0.008	1.090 ± 0.013	1.235 ± 0.023
Turmeric root powder (75 mg/ml)	1 ± 0	0.957 ± 0.006	0.903 ± 0.012	0.857 ± 0.016	0.823 ± 0.010	0.824 ± 0.014	0.852 ± 0.024	0.976 ± 0.014	0.993 ± 0.008	1.003 ± 0.006	1.049 ± 0.006	1.105 ± 0.005	1.134 ± 0.002	1.157 ± 0.010	1.174 ± 0.013	1.185 ± 0.014	2.816 ± 0.148
Garlic tablet (75 mg/ml)	1 ± 0	0.984 ± 0.006	0.979 ± 0.003	0.969 ± 0.007	0.977 ± 0.004	0.989 ± 0.008	0.992 ± 0.006	0.994 ± 0.008	1.001 ± 0.010	1.012 ± 0.010	1.044 ± 0.014	1.083 ± 0.010	1.103 ± 0.013	1.118 ± 0.011	1.125 ± 0.010	1.161 ± 0.018	1.731 ± 0.081
Ginger tablet (75 mg/ml)	1 ± 0	0.9851 ± 0.013	0.969 ± 0.005	0.955 ± 0.016	0.971 ± 0.006	0.976 ± 0.003	0.986 ± 0.006	0.985 ± 0.010	0.986 ± 0.012	0.989 ± 0.004	1.019 ± 0.006	1.026 ± 0.010	1.047 ± 0.011	1.059 ± 0.008	1.074 ± 0.012	1.089 ± 0.011	3.578 ± 0.210
Cinnamon tablet (75 mg/ml)	1 ± 0	0.983 ± 0.007	0.974 ± 0.003	0.965 ± 0.009	0.983 ± 0.005	0.988 ± 0.004	1.001 ± 0.007	1.018 ± 0.006	1.023 ± 0.005	1.029 ± 0.004	1.050 ± 0.006	1.066 ± 0.007	1.078 ± 0.009	1.091 ± 0.011	1.097 ± 0.011	1.114 ± 0.006	2.182 ± 0.074

Supplementary Table 3A (Cont.) The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain R11446.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Turmeric tablet (75 mg/ml)	1 ± 0	0.981 ± 0.007	0.961 ± 0.009	0.956 ± 0.008	0.976 ± 0.015	0.985 ± 0.013	0.992 ± 0.010	0.999 ± 0.005	1.001 ± 0.012	1.017 ± 0.009	1.065 ± 0.007	1.101 ± 0.013	1.116 ± 0.009	1.131 ± 0.009	1.154 ± 0.015	1.168 ± 0.007	1.952 ± 0.178
Artichoke (75 mg/ml)	1 ± 0	0.983 ± 0.005	0.974 ± 0.008	0.969 ± 0.006	0.965 ± 0.008	0.967 ± 0.006	0.967 ± 0.005	0.966 ± 0.017	0.966 ± 0.006	0.951 ± 0.008	0.950 ± 0.006	0.954 ± 0.008	0.956 ± 0.008	0.959 ± 0.008	0.959 ± 0.009	0.961 ± 0.008	0.958 ± 0.070
Aloe vera gel (32% w/v)	1 ± 0	0.984 ± 0.012	0.966 ± 0.007	0.963 ± 0.007	0.955 ± 0.006	0.946 ± 0.013	0.962 ± 0.017	0.980 ± 0.007	0.996 ± 0.011	1.022 ± 0.018	1.060 ± 0.012	1.067 ± 0.010	1.076 ± 0.014	1.091 ± 0.008	1.097 ± 0.012	1.109 ± 0.016	1.658 ± 0.173
Coconut oil (32% v/v)	1 ± 0	0.972 ± 0.008	0.955 ± 0.008	0.863 ± 0.028	0.837 ± 0.014	0.791 ± 0.007	0.765 ± 0.022	0.722 ± 0.016	0.716 ± 0.032	0.754 ± 0.009	0.788 ± 0.014	0.827 ± 0.031	0.906 ± 0.021	1.052 ± 0.079	1.165 ± 0.052	1.203 ± 0.017	1.663 ± 0.201
Peppermint oil (32% v/v)	1 ± 0	0.920 ± 0.008	0.822 ± 0.005	0.741 ± 0.007	0.650 ± 0.014	0.636 ± 0.019	0.600 ± 0.010	0.591 ± 0.013	0.627 ± 0.039	0.630 ± 0.037	0.668 ± 0.014	0.730 ± 0.034	0.753 ± 0.017	0.773 ± 0.006	0.782 ± 0.027	0.843 ± 0.032	1.175 ± 0.059
Allicin (75 mg/ml)	1 ± 0	0.993 ± 0.003	0.991 ± 0.008	0.991 ± 0.009	1.011 ± 0.004	1.017 ± 0.003	1.025 ± 0.003	1.039 ± 0.006	1.045 ± 0.006	1.052 ± 0.005	1.070 ± 0.011	1.086 ± 0.006	1.097 ± 0.011	1.096 ± 0.007	1.104 ± 0.008	1.105 ± 0.009	1.105 ± 0.027
trans-Cinnamaldehyde (32% v/v)	1 ± 0	0.978 ± 0.016	0.961 ± 0.012	0.950 ± 0.009	0.914 ± 0.008	0.927 ± 0.013	0.979 ± 0.006	0.990 ± 0.007	0.990 ± 0.012	1.004 ± 0.006	1.033 ± 0.009	1.041 ± 0.011	1.063 ± 0.010	1.082 ± 0.013	1.190 ± 0.013	1.221 ± 0.012	1.488 ± 0.054
Menthol (75 mg/ml)	1 ± 0	0.981 ± 0.004	0.977 ± 0.013	0.961 ± 0.008	0.986 ± 0.006	0.994 ± 0.006	1.006 ± 0.008	1.007 ± 0.010	1.023 ± 0.021	1.026 ± 0.007	1.035 ± 0.015	1.047 ± 0.008	1.066 ± 0.021	1.070 ± 0.014	1.102 ± 0.016	1.111 ± 0.022	1.513 ± 0.074
Zingerone (75 mg/ml)	1 ± 0	0.978 ± 0.006	0.968 ± 0.010	0.849 ± 0.024	0.763 ± 0.032	0.700 ± 0.017	0.684 ± 0.026	0.657 ± 0.021	0.584 ± 0.019	0.744 ± 0.009	0.883 ± 0.013	1.156 ± 0.020	1.249 ± 0.009	1.315 ± 0.017	1.394 ± 0.021	1.417 ± 0.024	1.843 ± 0.034
SDW	1 ± 0	0.972 ± 0.011	0.958 ± 0.011	0.956 ± 0.005	0.968 ± 0.015	0.969 ± 0.013	0.974 ± 0.008	0.977 ± 0.016	0.989 ± 0.010	0.989 ± 0.014	1.005 ± 0.015	1.029 ± 0.007	1.038 ± 0.021	1.056 ± 0.014	1.075 ± 0.020	1.112 ± 0.027	4.280 ± 0.314
DMSO (2.5% v/v)	1 ± 0	0.961 ± 0.013	0.957 ± 0.005	0.949 ± 0.008	0.959 ± 0.010	0.965 ± 0.007	0.976 ± 0.013	0.976 ± 0.009	0.980 ± 0.008	0.985 ± 0.019	1.002 ± 0.012	1.014 ± 0.011	1.036 ± 0.006	1.045 ± 0.007	1.076 ± 0.023	1.148 ± 0.034	3.669 ± 0.855
Fidaxomicin (16 mg/ml)	1 ± 0	0.974 ± 0.009	0.954 ± 0.007	0.946 ± 0.004	0.949 ± 0.007	0.953 ± 0.006	0.957 ± 0.006	0.958 ± 0.014	0.958 ± 0.010	0.962 ± 0.004	0.963 ± 0.010	0.965 ± 0.005	0.962 ± 0.010	0.961 ± 0.003	0.952 ± 0.015	0.952 ± 0.008	0.928 ± 0.025
Vancomycin (16 mg/ml)	1 ± 0	0.969 ± 0.011	0.957 ± 0.004	0.951 ± 0.007	0.951 ± 0.004	0.959 ± 0.015	0.959 ± 0.015	0.964 ± 0.008	0.966 ± 0.015	0.970 ± 0.021	0.967 ± 0.004	0.967 ± 0.003	0.964 ± 0.007	0.960 ± 0.002	0.953 ± 0.007	0.938 ± 0.013	0.924 ± 0.028

OD, optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour.

Supplementary Table 3B The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain R11446.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh onion bulb (25% v/v)	1 ± 0	0.976 ± 0.011	0.967 ± 0.009	0.963 ± 0.010	0.969 ± 0.009	0.969 ± 0.005	0.974 ± 0.012	0.982 ± 0.012	0.988 ± 0.015	0.994 ± 0.013	1.032 ± 0.007	1.051 ± 0.016	1.073 ± 0.027	1.091 ± 0.023	1.097 ± 0.008	1.108 ± 0.023	1.136 ± 0.024
Fresh onion bulb (12.5% v/v)	1 ± 0	0.975 ± 0.006	0.953 ± 0.005	0.953 ± 0.005	0.951 ± 0.008	0.953 ± 0.012	0.961 ± 0.014	0.961 ± 0.014	0.958 ± 0.009	0.958 ± 0.010	0.978 ± 0.009	0.990 ± 0.008	1.000 ± 0.014	1.010 ± 0.011	1.020 ± 0.011	1.035 ± 0.013	1.902 ± 0.253
Artichoke (37.5 mg/ml)	1 ± 0	0.990 ± 0.005	0.987 ± 0.004	0.986 ± 0.006	0.984 ± 0.005	0.982 ± 0.005	0.984 ± 0.008	0.987 ± 0.006	0.992 ± 0.004	0.997 ± 0.005	1.013 ± 0.005	1.019 ± 0.006	1.024 ± 0.008	1.019 ± 0.009	1.017 ± 0.009	11.019 ± 0.006	0.984 ± 0.028
Artichoke (18.8 mg/ml)	1 ± 0	0.987 ± 0.006	0.982 ± 0.007	0.978 ± 0.006	0.980 ± 0.009	0.980 ± 0.009	0.979 ± 0.008	0.980 ± 0.007	0.980 ± 0.006	0.980 ± 0.005	0.984 ± 0.005	0.990 ± 0.006	0.993 ± 0.010	0.993 ± 0.015	0.995 ± 0.005	0.993 ± 0.011	0.975 ± 0.011
Leptospermum honey A (8% w/v)	1 ± 0	0.982 ± 0.010	0.978 ± 0.008	0.975 ± 0.010	0.973 ± 0.005	0.970 ± 0.008	0.966 ± 0.020	0.960 ± 0.011	0.956 ± 0.007	0.958 ± 0.009	0.965 ± 0.010	0.966 ± 0.014	0.973 ± 0.007	0.974 ± 0.007	0.973 ± 0.009	0.970 ± 0.008	0.938 ± 0.009
Leptospermum honey A (4% w/v)	1 ± 0	0.979 ± 0.008	0.975 ± 0.007	0.973 ± 0.006	0.972 ± 0.009	0.972 ± 0.009	0.970 ± 0.009	0.965 ± 0.007	0.962 ± 0.012	0.957 ± 0.018	0.967 ± 0.022	0.972 ± 0.010	0.975 ± 0.007	0.980 ± 0.009	0.982 ± 0.006	0.983 ± 0.011	1.012 ± 0.058
Leptospermum honey B (8% w/v)	1 ± 0	0.987 ± 0.005	0.982 ± 0.004	0.980 ± 0.005	0.980 ± 0.005	0.980 ± 0.007	0.978 ± 0.010	0.971 ± 0.006	0.963 ± 0.009	0.967 ± 0.009	0.973 ± 0.010	0.978 ± 0.012	0.979 ± 0.010	0.980 ± 0.008	0.981 ± 0.006	0.980 ± 0.012	0.946 ± 0.019
Leptospermum honey B (4% w/v)	1 ± 0	0.984 ± 0.007	0.977 ± 0.004	0.973 ± 0.006	0.971 ± 0.008	0.971 ± 0.010	0.968 ± 0.008	0.963 ± 0.009	0.960 ± 0.011	0.965 ± 0.014	0.976 ± 0.010	0.989 ± 0.007	0.991 ± 0.010	0.983 ± 0.013	0.983 ± 0.030	0.978 ± 0.015	0.972 ± 0.033
Leptospermum honey C (8% w/v)	1 ± 0	0.989 ± 0.006	0.983 ± 0.004	0.979 ± 0.004	0.976 ± 0.007	0.971 ± 0.008	0.967 ± 0.004	0.959 ± 0.005	0.952 ± 0.007	0.943 ± 0.018	0.948 ± 0.015	0.955 ± 0.010	0.967 ± 0.009	0.969 ± 0.007	0.967 ± 0.014	0.962 ± 0.010	0.948 ± 0.015
Leptospermum honey C (4% w/v)	1 ± 0	0.988 ± 0.004	0.981 ± 0.006	0.977 ± 0.006	0.977 ± 0.005	0.986 ± 0.006	0.974 ± 0.006	0.972 ± 0.008	0.963 ± 0.006	0.955 ± 0.008	0.938 ± 0.009	0.942 ± 0.011	0.953 ± 0.016	0.964 ± 0.006	0.963 ± 0.010	0.973 ± 0.005	1.039 ± 0.082
Allicin (37.5 mg/ml)	1 ± 0	0.987 ± 0.005	0.972 ± 0.009	0.969 ± 0.005	0.970 ± 0.005	0.963 ± 0.006	0.963 ± 0.008	0.962 ± 0.008	0.960 ± 0.010	0.957 ± 0.012	0.963 ± 0.011	0.966 ± 0.014	0.968 ± 0.006	0.973 ± 0.008	0.978 ± 0.010	0.987 ± 0.009	1.440 ± 0.168
Allicin (18.8 mg/ml)	1 ± 0	0.981 ± 0.014	0.968 ± 0.007	0.966 ± 0.008	0.964 ± 0.006	0.962 ± 0.013	0.960 ± 0.015	0.961 ± 0.005	0.959 ± 0.009	0.963 ± 0.012	0.969 ± 0.006	0.976 ± 0.009	0.978 ± 0.007	0.980 ± 0.017	0.987 ± 0.006	1.001 ± 0.020	1.758 ± 0.241
SDW	1 ± 0	0.972 ± 0.011	0.958 ± 0.011	0.956 ± 0.005	0.968 ± 0.015	0.969 ± 0.013	0.974 ± 0.008	0.977 ± 0.016	0.989 ± 0.010	0.989 ± 0.014	1005 ± 0.015	1.029 ± 0.007	1.038 ± 0.021	1.056 ± 0.014	1.075 ± 0.020	1.112 ± 0.027	4.280 ± 0.314
DMSO (2.5% v/v)	1 ± 0	0.961 ± 0.013	0.957 ± 0.005	0.949 ± 0.008	0.959 ± 0.010	0.965 ± 0.007	0.976 ± 0.013	0.976 ± 0.009	0.980 ± 0.008	0.985 ± 0.019	1.002 ± 0.012	1.014 ± 0.011	1.036 ± 0.006	1.045 ± 0.007	1.076 ± 0.023	1.148 ± 0.034	3.669 ± 0.855
Fidaxomicin (8 mg/ml)	1 ± 0	0.964 ± 0.016	0.956 ± 0.008	0.953 ± 0.005	0.956 ± 0.004	0.955 ± 0.006	0.956 ± 0.010	0.967 ± 0.009	0.978 ± 0.012	0.986 ± 0.020	1.008 ± 0.022	1.008 ± 0.012	1.011 ± 0.009	1.016 ± 0.007	1.016 ± 0.008	1.027 ± 0.026	1.005 ± 0.021
Fidaxomicin (4 mg/ml)	1 ± 0	0.967 ± 0.009	0.959 ± 0.010	0.956 ± 0.008	0.958 ± 0.005	0.962 ± 0.006	0.962 ± 0.008	0.967 ± 0.012	0.975 ± 0.010	0.975 ± 0.012	0.989 ± 0.007	0.997 ± 0.011	0.997 ± 0.010	1.002 ± 0.008	1.006 ± 0.019	1.014 ± 0.021	1.016 ± 0.014

OD, optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour.

Supplementary Table 4A The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain ATCC 43598.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh garlic bulb (50% v/v)	1 ± 0	0.985 ± 0.009	0.968 ± 0.011	0.960 ± 0.007	0.958 ± 0.006	0.948 ± 0.009	0.942 ± 0.010	0.957 ± 0.003	0.964 ± 0.005	0.971 ± 0.011	0.992 ± 0.012	1.018 ± 0.007	1.043 ± 0.010	1.058 ± 0.013	1.069 ± 0.016	1.085 ± 0.030	1.326 ± 0.036
Fresh ginger rhizome (50% v/v)	1 ± 0	0.987 ± 0.004	0.981 ± 0.006	0.968 ± 0.009	0.957 ± 0.006	0.951 ± 0.010	0.972 ± 0.007	0.984 ± 0.006	1.002 ± 0.017	1.024 ± 0.008	1.078 ± 0.007	1.086 ± 0.011	1.102 ± 0.012	1.150 ± 0.014	1.164 ± 0.032	1.213 ± 0.015	1.490 ± 0.083
Fresh onion bulb (50% v/v)	1 ± 0	0.984 ± 0.003	0.980 ± 0.009	0.972 ± 0.006	0.961 ± 0.007	0.968 ± 0.010	0.971 ± 0.008	0.980 ± 0.009	0.977 ± 0.008	0.981 ± 0.017	0.980 ± 0.008	0.994 ± 0.014	0.986 ± 0.015	0.995 ± 0.014	0.978 ± 0.011	0.952 ± 0.006	0.960 ± 0.014
<i>Leptospermum</i> honey A (32% w/v)	1 ± 0	0.982 ± 0.10	0.977 ± 0.006	0.977 ± 0.006	0.984 ± 0.003	0.990 ± 0.10	0.996 ± 0.016	1.003 ± 0.011	1.010 ± 0.010	1.017 ± 0.008	1.032 ± 0.009	1.028 ± 0.018	1.016 ± 0.010	1.001 ± 0.005	0.992 ± 0.009	0.974 ± 0.020	0.941 ± 0.028
<i>Leptospermum</i> honey B (32% w/v)	1 ± 0	0.981 ± 0.004	0.976 ± 0.006	0.976 ± 0.008	0.977 ± 0.005	0.980 ± 0.005	0.982 ± 0.006	0.991 ± 0.006	0.998 ± 0.006	1.003 ± 0.005	1.021 ± 0.009	1.013 ± 0.018	1.000 ± 0.021	0.984 ± 0.006	0.965 ± 0.008	0.952 ± 0.016	0.931 ± 0.023
<i>Leptospermum</i> honey C (32% w/v)	1 ± 0	0.978 ± 0.008	0.973 ± 0.007	0.969 ± 0.006	0.982 ± 0.008	0.986 ± 0.015	0.983 ± 0.010	0.974 ± 0.005	0.976 ± 0.010	0.975 ± 0.006	0.979 ± 0.008	0.984 ± 0.008	0.989 ± 0.007	1.003 ± 0.005	1.012 ± 0.006	1.017 ± 0.008	0.992 ± 0.024
Garlic clove powder (75 mg/ml)	1 ± 0	0.979 ± 0.014	0.964 ± 0.006	0.959 ± 0.018	0.967 ± 0.012	0.970 ± 0.007	0.980 ± 0.010	1.002 ± 0.013	1.016 ± 0.009	1.031 ± 0.005	1.119 ± 0.028	1.152 ± 0.009	1.211 ± 0.015	1.275 ± 0.010	1.313 ± 0.016	1.349 ± 0.019	1.870 ± 0.098
Ginger rhizome powder (75 mg/ml)	1 ± 0	0.983 ± 0.004	0.973 ± 0.005	0.965 ± 0.008	0.954 ± 0.015	0.977 ± 0.005	0.979 ± 0.010	0.982 ± 0.011	0.990 ± 0.006	0.999 ± 0.006	1.011 ± 0.013	1.020 ± 0.014	1.031 ± 0.005	1.043 ± 0.006	1.060 ± 0.016	1.082 ± 0.009	1.541 ± 0.101
Cinnamon root powder (75 mg/ml)	1 ± 0	0.997 ± 0.007	0.992 ± 0.006	0.984 ± 0.006	0.988 ± 0.007	1.008 ± 0.008	1.017 ± 0.004	1.019 ± 0.004	1.043 ± 0.010	1.064 ± 0.013	1.074 ± 0.017	1.080 ± 0.007	1.091 ± 0.015	1.102 ± 0.005	1.117 ± 0.011	1.157 ± 0.024	1.805 ± 0.051
Turmeric root powder (75 mg/ml)	1 ± 0	0.969 ± 0.010	0.944 ± 0.013	0.871 ± 0.017	0.865 ± 0.007	0.904 ± 0.014	0.944 ± 0.009	0.969 ± 0.008	1.006 ± 0.025	1.022 ± 0.008	1.068 ± 0.011	1.080 ± 0.018	1.129 ± 0.026	1.143 ± 0.013	1.174 ± 0.018	1.181 ± 0.014	2.083 ± 0.057
Garlic tablet (75 mg/ml)	1 ± 0	0.975 ± 0.007	0.963 ± 0.009	0.951 ± 0.010	0.948 ± 0.006	0.965 ± 0.007	0.973 ± 0.011	0.987 ± 0.010	1.003 ± 0.012	1.010 ± 0.014	1.067 ± 0.022	1.077 ± 0.009	1.083 ± 0.013	1.091 ± 0.007	1.104 ± 0.012	1.108 ± 0.011	1.592 ± 0.132
Ginger tablet (75 mg/ml)	1 ± 0	0.985 ± 0.006	0.973 ± 0.006	0.937 ± 0.006	0.971 ± 0.008	0.982 ± 0.008	0.986 ± 0.020	0.990 ± 0.021	1.004 ± 0.006	1.007 ± 0.010	1.024 ± 0.011	1.031 ± 0.013	1.049 ± 0.014	1.063 ± 0.009	1.090 ± 0.011	1.117 ± 0.006	1.650 ± 0.018
Cinnamon tablet (75 mg/ml)	1 ± 0	0.979 ± 0.013	0.956 ± 0.008	0.939 ± 0.008	0.983 ± 0.010	0.991 ± 0.005	1.023 ± 0.016	1.028 ± 0.009	1.039 ± 0.007	1.071 ± 0.002	1.103 ± 0.001	1.105 ± 0.001	1.137 ± 0.011	1.174 ± 0.012	1.208 ± 0.013	1.236 ± 0.010	2.112 ± 0.099

Supplementary Table 4A (Cont.) The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain ATCC 43598.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Turmeric tablet (75 mg/ml)	1 ± 0	0.993 ± 0.092	0.978 ± 0.013	0.971 ± 0.009	0.964 ± 0.006	0.983 ± 0.010	0.986 ± 0.012	0.992 ± 0.010	0.990 ± 0.012	0.995 ± 0.010	1.015 ± 0.005	1.027 ± 0.007	1.043 ± 0.006	1.056 ± 0.007	1.075 ± 0.006	1.121 ± 0.021	1.565 ± 0.056
Artichoke (75 mg/ml)	1 ± 0	0.980 ± 0.006	0.969 ± 0.005	0.962 ± 0.007	0.956 ± 0.011	0.951 ± 0.006	0.947 ± 0.007	0.940 ± 0.005	0.935 ± 0.014	0.935 ± 0.005	0.927 ± 0.005	0.930 ± 0.009	0.931 ± 0.012	0.933 ± 0.013	0.935 ± 0.006	0.935 ± 0.008	0.924 ± 0.009
Aloe vera gel (32% w/v)	1 ± 0	0.970 ± 0.005	0.952 ± 0.009	0.939 ± 0.010	0.951 ± 0.009	0.966 ± 0.012	0.975 ± 0.008	0.985 ± 0.013	0.990 ± 0.012	1.009 ± 0.012	1.062 ± 0.009	1.071 ± 0.006	1.089 ± 0.009	1.116 ± 0.007	1.205 ± 0.009	1.264 ± 0.017	1.418 ± 0.047
Coconut oil (32% v/v)	1 ± 0	0.986 ± 0.009	0.966 ± 0.005	0.947 ± 0.018	0.915 ± 0.010	0.906 ± 0.012	0.899 ± 0.010	0.852 ± 0.030	0.811 ± 0.007	0.836 ± 0.011	0.924 ± 0.012	0.945 ± 0.017	0.970 ± 0.007	1.011 ± 0.018	1.114 ± 0.010	1.143 ± 0.016	1.656 ± 0.171
Peppermint oil (32% v/v)	1 ± 0	0.967 ± 0.014	0.923 ± 0.011	0.872 ± 0.009	0.856 ± 0.008	0.800 ± 0.006	0.771 ± 0.021	0.698 ± 0.008	0.666 ± 0.018	0.684 ± 0.015	0.770 ± 0.022	0.793 ± 0.014	0.840 ± 0.032	0.898 ± 0.011	0.919 ± 0.005	0.961 ± 0.009	1.303 ± 0.076
Allicin (75 mg/ml)	1 ± 0	0.988 ± 0.004	0.987 ± 0.003	0.983 ± 0.008	1.001 ± 0.010	1.014 ± 0.010	1.022 ± 0.009	1.032 ± 0.015	1.041 ± 0.007	1.048 ± 0.012	1.078 ± 0.010	1.082 ± 0.015	1.094 ± 0.008	1.097 ± 0.014	1.106 ± 0.005	1.111 ± 0.008	1.128 ± 0.064
trans-Cinnamaldehyde (32% v/v)	1 ± 0	0.944 ± 0.022	0.922 ± 0.010	0.886 ± 0.022	0.849 ± 0.022	0.874 ± 0.010	0.889 ± 0.007	0.904 ± 0.012	0.911 ± 0.010	0.923 ± 0.007	0.963 ± 0.007	0.977 ± 0.010	0.989 ± 0.007	1.009 ± 0.010	1.032 ± 0.006	1.063 ± 0.027	1.447 ± 0.061
Menthol (75 mg/ml)	1 ± 0	0.974 ± 0.011	0.966 ± 0.007	0.962 ± 0.005	0.949 ± 0.008	0.969 ± 0.12	0.977 ± 0.013	0.979 ± 0.010	0.985 ± 0.009	0.990 ± 0.014	1.019 ± 0.010	1.028 ± 0.006	1.039 ± 0.005	1.062 ± 0.011	1.076 ± 0.013	1.092 ± 0.016	1.460 ± 0.060
Zingerone (75 mg/ml)	1 ± 0	0.970 ± 0.012	0.952 ± 0.011	0.929 ± 0.0024	0.895 ± 0.0014	0.861 ± 0.010	0.846 ± 0.007	0.806 ± 0.024	0.749 ± 0.027	0.844 ± 0.067	0.993 ± 0.036	1.028 ± 0.020	1.058 ± 0.015	1.154 ± 0.027	1.256 ± 0.049	1.314 ± 0.014	1.915 ± 0.103
SDW	1 ± 0	0.973 ± 0.006	0.973 ± 0.006	0.968 ± 0.007	0.968 ± 0.005	0.972 ± 0.005	0.968 ± 0.007	0.977 ± 0.010	0.980 ± 0.017	0.980 ± 0.013	0.995 ± 0.008	1.012 ± 0.006	1.035 ± 0.019	1.055 ± 0.009	1.062 ± 0.017	1.081 ± 0.024	4.887 ± 0.226
DMSO (2.5% v/v)	1 ± 0	0.969 ± 0.004	0.964 ± 0.005	0.963 ± 0.004	0.954 ± 0.007	0.966 ± 0.005	0.964 ± 0.008	0.966 ± 0.012	0.959 ± 0.005	0.956 ± 0.007	0.976 ± 0.008	0.990 ± 0.013	0.998 ± 0.008	1.017 ± 0.011	1.034 ± 0.008	1.065 ± 0.011	3.674 ± 0.511
Fidaxomicin (16 mg/ml)	1 ± 0	0.975 ± 0.006	0.971 ± 0.004	0.968 ± 0.011	0.963 ± 0.012	0.960 ± 0.007	0.961 ± 0.010	0.962 ± 0.007	0.961 ± 0.006	0.964 ± 0.006	0.964 ± 0.004	0.964 ± 0.003	0.958 ± 0.010	0.957 ± 0.006	0.944 ± 0.011	0.932 ± 0.012	0.913 ± 0.014
Vancomycin (16 mg/ml)	1 ± 0	0.975 ± 0.008	0.959 ± 0.005	0.959 ± 0.004	0.954 ± 0.008	0.952 ± 0.004	0.952 ± 0.014	0.950 ± 0.010	0.947 ± 0.005	0.946 ± 0.006	0.961 ± 0.007	0.963 ± 0.010	0.968 ± 0.004	0.970 ± 0.010	0.970 ± 0.007	0.964 ± 0.006	0.958 ± 0.012

OD, optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour.

Supplementary Table 4B The normalized OD₆₀₀ ratios representing the effect of natural products on germination and outgrowth of *C. difficile* spores for strain ATCC 43598.

Agents	OD ₆₀₀ (t)/OD ₆₀₀ (t ₀) ± SD																
	0	5 min	10 min	15 min	20 min	25 min	30 min	40 min	50 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
Fresh onion bulb (25% v/v)	1 ± 0	0.976 ± 0.011	0.967 ± 0.009	0.963 ± 0.010	0.969 ± 0.009	0.969 ± 0.005	0.974 ± 0.012	0.982 ± 0.012	0.988 ± 0.015	0.994 ± 0.013	1.032 ± 0.007	1.051 ± 0.016	1.073 ± 0.027	1.091 ± 0.023	1.097 ± 0.008	1.108 ± 0.023	1.136 ± 0.024
Fresh onion bulb (12.5% v/v)	1 ± 0	0.975 ± 0.006	0.953 ± 0.005	0.953 ± 0.005	0.951 ± 0.008	0.953 ± 0.012	0.961 ± 0.014	0.961 ± 0.014	0.958 ± 0.009	0.958 ± 0.010	0.978 ± 0.009	0.990 ± 0.008	1.000 ± 0.014	1.010 ± 0.011	1.020 ± 0.011	1.035 ± 0.013	1.902 ± 0.253
Artichoke (37.5 mg/ml)	1 ± 0	0.990 ± 0.005	0.987 ± 0.004	0.986 ± 0.006	0.984 ± 0.005	0.982 ± 0.005	0.984 ± 0.008	0.987 ± 0.006	0.992 ± 0.004	0.997 ± 0.005	1.013 ± 0.005	1.019 ± 0.006	1.024 ± 0.008	1.019 ± 0.009	1.017 ± 0.009	1.1019 ± 0.006	0.984 ± 0.028
Artichoke (18.8 mg/ml)	1 ± 0	0.987 ± 0.006	0.982 ± 0.007	0.978 ± 0.006	0.980 ± 0.009	0.980 ± 0.009	0.979 ± 0.008	0.980 ± 0.007	0.980 ± 0.006	0.980 ± 0.005	0.984 ± 0.005	0.990 ± 0.006	0.993 ± 0.010	0.993 ± 0.015	0.995 ± 0.005	0.993 ± 0.011	0.975 ± 0.011
<i>Leptospermum</i> honey A (8% w/v)	1 ± 0	0.982 ± 0.010	0.978 ± 0.008	0.975 ± 0.010	0.973 ± 0.005	0.970 ± 0.008	0.966 ± 0.020	0.960 ± 0.011	0.956 ± 0.007	0.958 ± 0.009	0.965 ± 0.010	0.966 ± 0.014	0.973 ± 0.007	0.974 ± 0.007	0.973 ± 0.009	0.970 ± 0.008	0.938 ± 0.009
<i>Leptospermum</i> honey A (4% w/v)	1 ± 0	0.979 ± 0.008	0.975 ± 0.007	0.973 ± 0.006	0.972 ± 0.009	0.972 ± 0.009	0.970 ± 0.009	0.965 ± 0.007	0.962 ± 0.012	0.957 ± 0.018	0.967 ± 0.022	0.972 ± 0.010	0.975 ± 0.007	0.980 ± 0.009	0.982 ± 0.006	0.983 ± 0.011	1.012 ± 0.058
<i>Leptospermum</i> honey B (8% w/v)	1 ± 0	0.987 ± 0.005	0.982 ± 0.004	0.980 ± 0.005	0.980 ± 0.005	0.980 ± 0.007	0.978 ± 0.010	0.971 ± 0.006	0.963 ± 0.009	0.967 ± 0.009	0.973 ± 0.010	0.978 ± 0.012	0.979 ± 0.010	0.980 ± 0.008	0.981 ± 0.006	0.980 ± 0.012	0.946 ± 0.019
<i>Leptospermum</i> honey B (4% w/v)	1 ± 0	0.984 ± 0.007	0.977 ± 0.004	0.973 ± 0.006	0.971 ± 0.008	0.971 ± 0.010	0.968 ± 0.008	0.963 ± 0.009	0.960 ± 0.011	0.965 ± 0.014	0.976 ± 0.010	0.989 ± 0.007	0.991 ± 0.010	0.983 ± 0.013	0.983 ± 0.030	0.978 ± 0.015	0.972 ± 0.033
<i>Leptospermum</i> honey C (8% w/v)	1 ± 0	0.989 ± 0.006	0.983 ± 0.004	0.979 ± 0.004	0.976 ± 0.007	0.971 ± 0.008	0.967 ± 0.004	0.959 ± 0.005	0.952 ± 0.007	0.943 ± 0.018	0.948 ± 0.015	0.955 ± 0.010	0.967 ± 0.009	0.969 ± 0.007	0.967 ± 0.014	0.962 ± 0.010	0.948 ± 0.015
<i>Leptospermum</i> honey C (4% w/v)	1 ± 0	0.988 ± 0.004	0.981 ± 0.006	0.977 ± 0.006	0.977 ± 0.005	0.986 ± 0.006	0.974 ± 0.006	0.972 ± 0.008	0.963 ± 0.006	0.955 ± 0.008	0.938 ± 0.009	0.942 ± 0.011	0.953 ± 0.016	0.964 ± 0.006	0.963 ± 0.010	0.973 ± 0.005	1.039 ± 0.082
Allicin (37.5 mg/ml)	1 ± 0	0.987 ± 0.005	0.972 ± 0.009	0.969 ± 0.005	0.970 ± 0.005	0.963 ± 0.006	0.963 ± 0.008	0.962 ± 0.008	0.960 ± 0.010	0.957 ± 0.012	0.963 ± 0.011	0.966 ± 0.014	0.968 ± 0.006	0.973 ± 0.008	0.978 ± 0.010	0.987 ± 0.009	1.440 ± 0.168
Allicin (18.8 mg/ml)	1 ± 0	0.981 ± 0.014	0.968 ± 0.007	0.966 ± 0.008	0.964 ± 0.006	0.962 ± 0.013	0.960 ± 0.015	0.961 ± 0.005	0.959 ± 0.009	0.963 ± 0.012	0.969 ± 0.006	0.976 ± 0.009	0.978 ± 0.007	0.980 ± 0.017	0.987 ± 0.006	1.001 ± 0.020	1.758 ± 0.241
SDW	1 ± 0	0.972 ± 0.011	0.958 ± 0.011	0.956 ± 0.005	0.968 ± 0.015	0.969 ± 0.013	0.974 ± 0.008	0.977 ± 0.016	0.989 ± 0.010	0.989 ± 0.014	1.005 ± 0.015	1.029 ± 0.007	1.038 ± 0.021	1.056 ± 0.014	1.075 ± 0.020	1.112 ± 0.027	4.280 ± 0.314
DMSO (2.5% v/v)	1 ± 0	0.961 ± 0.013	0.957 ± 0.005	0.949 ± 0.008	0.959 ± 0.010	0.965 ± 0.007	0.976 ± 0.013	0.976 ± 0.009	0.980 ± 0.008	0.985 ± 0.019	1.002 ± 0.012	1.014 ± 0.011	1.036 ± 0.006	1.045 ± 0.007	1.076 ± 0.023	1.148 ± 0.034	3.669 ± 0.855
Fidaxomicin (8 mg/ml)	1 ± 0	0.964 ± 0.016	0.956 ± 0.008	0.953 ± 0.005	0.956 ± 0.004	0.955 ± 0.006	0.956 ± 0.010	0.967 ± 0.009	0.978 ± 0.012	0.986 ± 0.020	1.008 ± 0.022	1.008 ± 0.012	1.011 ± 0.009	1.016 ± 0.007	1.016 ± 0.008	1.027 ± 0.026	1.005 ± 0.021
Fidaxomicin (4 mg/ml)	1 ± 0	0.967 ± 0.009	0.959 ± 0.010	0.956 ± 0.008	0.958 ± 0.005	0.962 ± 0.006	0.962 ± 0.008	0.967 ± 0.012	0.975 ± 0.010	0.975 ± 0.012	0.989 ± 0.007	0.997 ± 0.011	0.997 ± 0.010	1.002 ± 0.008	1.006 ± 0.019	1.014 ± 0.021	1.016 ± 0.014

OD, optical density; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; t, time; min, minute; h, hour.

Appendix II Supplementary data for Chapter 5

Supplementary Figure 1 Viability of (A) Vero cells and (B) HT-29 cells determined by the neutral red uptake assay. (Cells were pre-incubated with treatments 2 h prior to addition of toxin filtrate).

Statistical significance: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to culture filtrate control.

Supplementary Figure 2 Viability of (A) Vero cells and (B) HT-29 cells determined by the neutral red uptake assay. (Culture filtrates and treatments were added to the cells without prior incubation).

Statistical significance: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to culture filtrate control.

ORIGINAL ARTICLE

Antimicrobial activity of natural products against *Clostridium difficile* in vitro

N. Roshan¹, T.V. Riley^{1,2,3,4} and K.A. Hammer¹ ¹ School of Biomedical Sciences (M504), The University of Western Australia, Crawley, WA, Australia² Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia³ School of Veterinary & Life Sciences, Murdoch University, Murdoch, WA, Australia⁴ School of Medical & Health Sciences, Edith Cowan University, Joondalup, WA, Australia**Keywords**

alternative therapy, CDI, cinnamon, garlic, peppermint oil.

CorrespondenceKatherine A. Hammer, School of Biomedical Sciences (M504), The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia.
E-mail: katherine.hammer@uwa.edu.au

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Abstract**Aims:** To investigate the antimicrobial activity of various natural products against *Clostridium difficile* in vitro.**Methods and Results:** The antibacterial activity of 20 natural products was determined by the agar well diffusion and broth microdilution assays against four *C. difficile* strains, three comparator organisms and four gastrointestinal commensal organisms. Of the raw natural products, garlic juice had the highest activity. The most active processed products were peppermint oil and the four pure compounds *trans*-cinnamaldehyde, allicin, menthol and zingerone. Furthermore, *Bacteroides* species had similar susceptibility to *C. difficile* to most natural products; however, *Lactobacillus casei* was less susceptible. The combined effect of natural products with vancomycin or metronidazole was determined using the conventional checkerboard titration method and the fractional inhibitory concentration index was calculated. The results showed a possible synergism between *trans*-cinnamaldehyde and vancomycin and partial synergy between *trans*-cinnamaldehyde and metronidazole.**Conclusions:** The study indicates a range of antimicrobial activity of natural products against *C. difficile* and suggests that they may be useful as alternative or complementary treatments for *C. difficile* infection (CDI), particularly as most are able to be given orally.**Significance and Impact of the Study:** This study encourages further investigation of natural products for treatment of CDI.**Introduction**

Clostridium difficile is a Gram-positive, spore-forming anaerobic bacterium that causes disease ranging from antibiotic-associated diarrhoea to life threatening pseudomembranous colitis (McDonald *et al.* 2005). *Clostridium difficile* is one of the main causes of nosocomial diarrhoea and commonly affects both hospitalized patients and people in the community. The pathogenesis of *C. difficile* infection (CDI) is attributed to the production of the two toxins toxin A and toxin B (Voth and Ballard 2005). There is also a third toxin named binary toxin that is produced by some strains of *C. difficile*,

including the hypervirulent NAP1/027 epidemic strain. However, there is still little known about the role of this toxin in virulence (Gerding *et al.* 2014).

Various factors increase the risk of acquiring CDI including exposure to multiple antibiotics, advanced age, prolonged hospitalization, long-term stay in health-care facilities, underlying diseases, immunosuppression and the use of gastric acid inhibitors (Owens *et al.* 2008; Henrich *et al.* 2009). The usual treatment for CDI is antibiotics, which is somewhat of a paradox given that the most important risk factor for the disease is exposure to antibiotics. Metronidazole has been the antibiotic of choice for treating mild to moderate CDI and

vancomycin is recommended as an initial therapy for severe disease (Hedge *et al.* 2008; Cheng *et al.* 2011). However, there is concern about the emergence of antibiotic resistance resulting in treatment failure (Koo *et al.* 2010). Issues associated with the use of conventional therapies for CDI treatment prompt investigating other possible treatment strategies such as natural remedies. Medicinal plants are the primary source of treatment in rural areas of most developing countries (Num and Useh 2014). Complementary and natural medicines are considered by many consumers as a safer and less toxic option compared to most conventional therapies. There are a wide range of medicinal plants used throughout the world of which only a small number have been studied (World Health Organisation, 2014). Thus, this study aimed to investigate the antimicrobial activity of a range of natural products against *C. difficile* *in vitro*.

Materials and methods

Micro-organisms

Organisms were obtained from the School of Biomedical Sciences at The University of Western Australia and from PathWest Laboratory Medicine, WA. Strains were *C.*

difficile ATCC 700057, *C. difficile* ribotype UK 027 NCTC 13366, *C. difficile* ribotype UK 014 (clinical isolate), *C. difficile* ribotype UK 017 ATCC 43598, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Clostridium perfringens* ATCC 13124, *Bacteroides fragilis* NCTC 9343, *B. vulgatus* NCTC 10583, *B. thetaiotaomicron* 5018 (clinical isolate), *Lactobacillus casei* Q328 (clinical isolate) and *L. casei* M2971 (clinical isolate).

Natural products

Natural products were selected for investigation based on factors such as historical evidence of use, current popularity or feasibility. The products were categorized into two broad groups; one group representing raw products that people consumed as food and the other group representing processed products that are taken as health supplements.

The raw products ($n = 8$) were purchased from a supermarket in Perth, Western Australia (WA) (Table 1). The WA-produced manuka honey was provided by Capilano Company, Bayswater, WA. Processed products ($n = 12$) were purchased from pharmacies and health food stores in Perth, WA, or were purchased online (Table 2).

Table 1 Screening raw natural products for antimicrobial activity by agar diffusion against three *Clostridium difficile* isolates and three comparator isolates

Raw product (juice or dried powder)	Solvent	Mean zone of inhibition in mm ($\pm$ SD) [†]					
		<i>Staphylococcus aureus</i> ATCC 29213	<i>Escherichia coli</i> ATCC 25922	<i>Clostridium perfringens</i> ATCC 13124	<i>C. difficile</i> ATCC 13366	<i>C. difficile</i> ribotype UK 014	<i>C. difficile</i> ATCC 700057
Onion juice (100% v/v)	–	10.7 $\pm$ 0.6	–	15.3 $\pm$ 0.6	10.3 $\pm$ 0.6	–	11.0 $\pm$ 0.0
Garlic juice (100% v/v)	–	42.4 $\pm$ 0.5	32.3 $\pm$ 0.9	28.7 $\pm$ 0.6	27.0 $\pm$ 1.0	26.1 $\pm$ 1.0	27.7 $\pm$ 0.6
Ginger juice (100% v/v)	–	–	–	–	–	–	–
WA Manuka honey (50% w/v)	SDW	15.9 $\pm$ 0.7	10.7 $\pm$ 0.4	16.5 $\pm$ 0.5	11.4 $\pm$ 0.5	10.5 $\pm$ 0.5	15.3 $\pm$ 0.6
Garlic powder (20% w/v)	SDW	32.3 $\pm$ 1.1	23.4 $\pm$ 0.5**	21.5 $\pm$ 0.5**	22.0 $\pm$ 0.9**	19.1 $\pm$ 0.4***	21.0 $\pm$ 0.9*
	20% DMSO	33.1 $\pm$ 1.2	25.4 $\pm$ 0.5	26.5 $\pm$ 0.7	26.6 $\pm$ 0.6	26.8 $\pm$ 0.7	24.4 $\pm$ 0.5
Ginger powder (20% w/v)	SDW	–*	–	–*	–*	–*	–*
	20% DMSO	12.0 $\pm$ 0.9	–	10.2 $\pm$ 0.7	9.6 $\pm$ 0.5	10.0 $\pm$ 0.0	10.9 $\pm$ 0.5
Cinnamon powder (20% w/v)	SDW	16.2 $\pm$ 0.7	–*	12.0 $\pm$ 1.0**	17.8 $\pm$ 0.7**	18.6 $\pm$ 0.7*	17.3 $\pm$ 0.6**
	20% DMSO	16.2 $\pm$ 0.7	11.1 $\pm$ 0.5	16.1 $\pm$ 0.9	20.9 $\pm$ 0.9	21.0 $\pm$ 0.7	20.4 $\pm$ 0.5
Turmeric powder (20% w/v)	SDW	–*	–*	10.0 $\pm$ 0.9	–*	–*	–*
	20% DMSO	9.0 $\pm$ 0.0	11.0 $\pm$ 0.9	10.0 $\pm$ 0.0	9.2 $\pm$ 0.7	10.0 $\pm$ 0.0	11 $\pm$ 1.0
Trimethoprim 5 mg/disc (positive control)	–	26.1 $\pm$ 0.8	26 $\pm$ 1.0	ND	ND	ND	ND
Vancomycin 30 μ g/disc (positive control)	–	ND	ND	22.5 $\pm$ 1.3	30.3 $\pm$ 0.7	28.3 $\pm$ 0.9	28.7 $\pm$ 0.9

SD, standard deviation; DMSO, dimethyl sulfoxide; SDW, sterile distilled water; ND, not done.

SDW and 20% DMSO were used as negative controls (no zone of inhibition was observed with 20% DMSO or SDW).

[†]P-values indicating the difference in activity of each product dissolved in either solvent against different isolates: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

If no zone of inhibition was observed, an 8-mm zone size was assumed to enable calculating the P-value.

Table 2 Screening processed natural products for antimicrobial activity by agar diffusion against three *Clostridium difficile* isolates and three comparator isolates

Processed product (capsules, tablets, oils and gels)	Solvent	Mean zone of inhibition in mm ($\pm$ SD) [†]					
		<i>Staphylococcus aureus</i> ATCC 29213	<i>Escherichia coli</i> ATCC 25922	<i>C. perfringens</i> ATCC 13124	<i>C. difficile</i> ATCC 13366	<i>C. difficile</i> ribotype UK 014	<i>C. difficile</i> ATCC 700057
Garlic tablet (20% w/v)	SDW	20.4 $\pm$ 0.8*	14.7 $\pm$ 0.6*	—*	14.7 $\pm$ 0.6	14.0 $\pm$ 0.9	15.4 $\pm$ 0.5
	20% DMSO	21.9 $\pm$ 0.9	17.1 $\pm$ 0.9	15.5 $\pm$ 0.5	16.1 $\pm$ 0.9	14.1 $\pm$ 0.2	15.8 $\pm$ 0.2
Ginger tablet (20% w/v)	SDW	—*	—*	—*	—	—*	—*
	20% DMSO	9.3 $\pm$ 0.6	10.0 $\pm$ 0.2	10.0 $\pm$ 0.0	—	11.0 $\pm$ 0.0	9.7 $\pm$ 0.6
Cinnamon tablet (20% w/v)	SDW	—	—	10.7 $\pm$ 0.6	10.0 $\pm$ 0.9*	—	10.2 $\pm$ 0.7
	20% DMSO	—	—	11.6 $\pm$ 0.5	11.6 $\pm$ 0.5	11.9 $\pm$ 0.9	11.0 $\pm$ 1.3
Turmeric tablet (20% w/v)	SDW	—*	—*	—*	11.0 $\pm$ 0.0*	11.3 $\pm$ 0.3*	10.9 $\pm$ 0.9
	20% DMSO	9.0 $\pm$ 0.0	11.0 $\pm$ 0.2	9.0 $\pm$ 0.0	11.7 $\pm$ 0.6	12.5 $\pm$ 0.5	11.3 $\pm$ 0.6
Artichoke capsule (20% w/v)	SDW	11.7 $\pm$ 0.4	—	12.7 $\pm$ 0.6	13.1 $\pm$ 0.2	13.4 $\pm$ 0.5	12.7 $\pm$ 0.6
	20% DMSO	12.0 $\pm$ 0.9	—	13.3 $\pm$ 0.6	13.8 $\pm$ 0.3	13.9 $\pm$ 0.2	13.7 $\pm$ 0.4
Aloe Vera gel (50% w/v)	SDW	—	9.0 $\pm$ 0.0	15.3 $\pm$ 0.6**	14.2 $\pm$ 0.3	14.1 $\pm$ 0.2	15.7 $\pm$ 0.6**
	20% DMSO	—	9.0 $\pm$ 0.0	19.2 $\pm$ 0.3	15.0 $\pm$ 0.9	14.4 $\pm$ 0.2	18.8 $\pm$ 0.4
Coconut oil (100% v/v)	—	—	—	—	—	—	—
Peppermint oil (100% v/v)	—	10.6 $\pm$ 0.5	12.4 $\pm$ 0.8	11.6 $\pm$ 0.6	11.9 $\pm$ 0.2	11.6 $\pm$ 0.5	12.3 $\pm$ 0.6

SD, standard deviation; DMSO, dimethyl sulfoxide; SDW, sterile distilled water; ND, not done.

SDW and 20% DMSO were used as negative controls (no zone of inhibition was observed with 20% DMSO or SDW).

[†]P-values indicating the difference in activity of each product dissolved in either solvents against different isolates: *P < 0.05, **P < 0.01.

If no zone of inhibition was observed, an eight mm zone size was assumed to enable calculating the P-value.

To obtain preparations for testing, raw products such as garlic, ginger and onion were washed with sterile distilled water (SDW), peeled and crushed using a mortar and pestle or a grinder. They were then filtered through cheesecloth to obtain the juice. The honey was prepared (w/v) in SDW and solutions were held at 37°C for 15 min to aid mixing. For the agar well diffusion assay, the dried powdered spices were suspended in either SDW or 20% dimethyl sulfoxide (DMSO) and were shaken gently at room temperature overnight after which, the spice suspensions were centrifuged and the supernatant retained for testing. One portion of each sample was passed through a 0.2- μ m pore filter to remove any contamination and the remainder was tested without filtration. The processed products were either used neat or were suspended in 20% DMSO. Since the processed products were assumed to contain no microbial contamination as they would have had gone through a sterilization process, they were not filtered. For the remaining assays including broth microdilution experiments, products in the form of powder or oil were primarily dissolved in 20% DMSO. Further dilutions were performed in SDW for all the products.

Inoculum preparation

Inocula were prepared by culturing anaerobic bacteria on prereduced *Brucella* agar supplemented with 5 μ g hemin,

1 μ g vitamin K₁ and 5% (v/v) laked sheep blood. Plates were incubated for 24–48 h at 35°C in an anaerobic hamber (Don Whitley Scientific, London, UK), while aerobic bacteria were cultured on blood agar for 18–24 h at 35°C. Growth was suspended in prereduced 0.85% saline for anaerobic bacteria and nonreduced 0.85% saline for aerobic bacteria and adjusted to be equivalent to a 0.5 McFarland standard, unless otherwise stated. The 0.5 McFarland standard was equivalent to approximately 1.4×10^7 CFU per ml for *C. difficile* based on preliminary data and Clinical and Laboratory Standards Institute (CLSI) guidelines, and 1×10^8 CFU per ml for the remaining organisms.

Agar well diffusion assay

All natural products were screened for activity against three reference *C. difficile* isolates and three comparator strains using an agar well diffusion assay (Tables 1 and 2). Prereduced *Brucella* agar or MHA was lawn inoculated with the inocula. Wells (8 mm) were cut into each inoculated agar plate and a 100- μ l aliquot of each unfiltered solution was pipetted into each well. The raw products were also used to assess the effect of filtration on antimicrobial activity. Tests with anaerobic bacteria were incubated in an anaerobic chamber at 35°C for 48 h and tests with aerobic bacteria were incubated at 35°C for 18–24 h. After incubation, zones of growth inhibition were measured to the nearest millimetre.

Four additional processed products, *trans*-cinnamaldehyde, allicin, menthol and zingerone were also tested in the remainder of the antimicrobial activity assays.

Broth microdilution assay

The minimum inhibitory concentration (MIC) of natural products against anaerobic and aerobic bacteria was determined according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2009, 2015). Briefly, each product was serially diluted twofold across a 96-well tray. The final inocula concentration for anaerobes and aerobes was approximately 1×10^6 and 5×10^5 CFU per ml respectively. Tests with anaerobic bacteria were conducted in supplemented *Brucella* broth incubated for 24–48 h and tests with aerobes were performed in Mueller–Hinton broth incubated for 18–24 h. Tests with *L. casei* were conducted in MRS broth and incubated anaerobically at 35°C for 24–48 h. MICs for all organisms were determined visually using a reading mirror as the lowest concentration of product preventing growth and resulting in no turbidity. The minimum bactericidal concentration (MBC) was determined by subculturing 10- μ l aliquots from nonturbid wells onto blood agar. After incubating the blood agar plates, colonies were counted and the lowest concentration showing a 99.9% reduction in the initial inoculum was recorded as the MBC. Also serial twofold dilutions of 20% DMSO were prepared and tested against all the micro-organisms in each experiment.

Checkerboard testing to detect synergy or antagonism

The checkerboard method was performed to determine the effect of combining natural therapeutic agents with the antibiotics vancomycin or metronidazole against *C. difficile* ATCC 700057 and *C. difficile* NCTC 13366. One agent was serially diluted across each row of a 96-well microtitre tray and serial dilutions of the second agent were then added to each column. Therefore, each row and column contained a fixed concentration of one agent and decreasing concentration of the other agent. Each tray contained one row and one column with serial dilution of each agent alone. The remainder of the assay was performed as described for the broth microdilution assay. The fractional inhibitory concentration (FIC) was calculated for each therapeutic agent in each combination. The following equation was used to calculate the FIC index: $FIC_{\text{antibiotic}} = MIC_{\text{antibiotic combination}} / MIC_{\text{antibiotic alone}}$, $FIC_{\text{natural product}} = MIC_{\text{natural product combination}} / MIC_{\text{natural product alone}}$ and $FIC_{\text{index}} = FIC_{\text{antibiotic}} + FIC_{\text{natural product}}$. The FIC index was interpreted as synergistic ($FIC \leq 0.5$), no interaction ($FIC > 0.5-4.0$) or antagonistic ($FIC > 4.0$)

(Botelho 2000). An isobologram was also generated for the combined amount of two agents required to produce the MIC.

Statistical analysis

All assays were repeated at least three times. The mean and standard deviation were calculated for zones of growth inhibition obtained by the agar well diffusion assay and a modal value was determined for MIC and MBC values obtained by the broth microdilution assays. The Student's two-tailed *t*-test, assuming unequal variance was used to determine whether there was a significant difference between two sets of data. *P*-values of <0.05 were considered significant.

Results

The natural products were screened for activity at their highest possible concentration using an agar well diffusion assay. Zones of inhibition were observed for 14 of the 16 products. Of the eight raw products, all except ginger juice (100% v/v) showed activity (Table 1). The most active agent in the raw product group was the 100% v/v garlic juice. Of the eight processed preparations, all except coconut oil capsules showed activity (Table 2). The garlic tablet was the most active and ginger tablet was the least active of the seven processed products. Moreover, a 20% DMSO control was included in each experiment and no zones of inhibition were observed. In general, products dissolved better in 20% DMSO and showed larger zones than when dissolved in SDW. For instance, both ginger powder and tablets did not show any zones of inhibition when SDW was used as a solvent; similarly, 20% w/v turmeric powder and tablet showed minimal activity in water with zones ranging from 10.0 to 11.3 mm. In general, larger zones of inhibition were observed for both ginger (9.3–12.0 mm) and turmeric products (9.0–12.5 mm) when dissolved in 20% DMSO (Tables 1 and 2). These larger zones may be due to increase in solubility of products in the agar as a result of DMSO.

Since the raw products were not sterile and may have contained organisms that would interfere with the results of assay, the effect of filtration on the antimicrobial activity of raw products against *C. difficile* isolates and comparators was assessed. Filtration had no significant effect on the size of inhibition zones ($P < 0.05$) (data not shown). Therefore, to avoid contamination, raw products were filter sterilized for the remaining antimicrobial activity assays.

Using the broth microdilution assay approximately 60% of natural agents at their highest concentrations showed inhibitory effects against *C. difficile* isolates and

three comparator micro-organisms (Tables 3 and 4). Among the raw products, garlic juice and garlic powder showed the highest inhibitory effect with MICs of 0.8% v/v for garlic juice and 9.4 mg ml⁻¹ for garlic powder against *C. difficile* isolates. This corresponded to the results from the agar well diffusion screening method, where garlic juice produced the largest zone of inhibition followed by garlic powder. However, the only raw product to show bactericidal activity against *C. difficile* isolates was cinnamon powder. Of the processed products, approximately 67% showed some inhibitory effect at their highest concentrations with the four pure compounds allcin, *trans*-cinnamaldehyde, menthol and zingerone showing the great activity. None of the garlic preparations showed any bactericidal effect against *C. difficile*. On the other hand, inhibitory and bactericidal activities were observed with both cinnamon powder and *trans*-cinnamaldehyde (a major constituent of cinnamon). Peppermint oil also displayed both inhibitory and bactericidal effects at 8% v/v against *C. difficile* strains and

similarly menthol, the main ingredient of peppermint oil, showed MICs of 9.4 mg ml⁻¹ and MBC values of 18.8 mg ml⁻¹ against *C. difficile*. Interestingly, zingerone (in contrast with both ginger juice and ginger powder) showed reasonable activity against *C. difficile* isolates with MIC and MBC values of 0.4 and 18.8 mg ml⁻¹ respectively. Manuka honey, artichoke capsules and aloe vera gel also had some inhibitory effect within the range of concentrations used; however, the aloe vera gel contained 1% preservatives such as disodium edetate that may have contributed to its antimicrobial activity. For a control, serial twofold dilutions of 20% DMSO were tested against all the micro-organisms. While 10% DMSO showed some inhibitory and bactericidal activity, no effect was observed with 5% DMSO or lower. For either MIC or MBCs of 150 mg ml⁻¹ with 20% DMSO, the test was repeated in 10% DMSO instead of 20% DMSO to remove any DMSO effect.

The broth microdilution assay was also used to examine the natural products for activity against commensal

Table 3 Susceptibility of micro-organisms to raw natural products determined by the broth microdilution method

			Micro-organisms						
			<i>Staphylococcus aureus</i> ATCC 29213	<i>Escherichia coli</i> ATCC 25922	<i>Clostridium perfringens</i> ATCC 13124	<i>Clostridium difficile</i> ATCC 13366	<i>C. difficile</i> ribotype UK 014	<i>C. difficile</i> ATCC 43598	<i>C. difficile</i> ATCC 700057
Raw product	Solvent								
Garlic juice % (v/v)	MIC	SDW	0.8	0.8	0.8	0.8	0.8	0.8	0.8
	MBC		12.5	6.3	6.3	50	50	50	50
Ginger juice % (v/v)	MIC	SDW	>50	>50	>50	>50	>50	>50	>50
	MBC		>50	>50	>50	>50	>50	>50	>50
Onion juice % (v/v)	MIC	SDW	>50	>50	25	>50	>50	>50	>50
	MBC		>50	>50	>50	>50	>50	>50	>50
Manuka honey % (w/v)	MIC	SDW	32	32	32	32	32	32	32
	MBC		>32	>32	>32	>32	>32	>32	>32
Garlic powder (mg ml ⁻¹)	MIC	SDW	9.4	4.7	18.8	9.4	9.4	9.4	9.4
	MBC		37.5	18.8	37.5	>150	>150	>150	>150
	MIC	20% DMSO	4.7	4.7	9.4	9.4	9.4	9.4	9.4
	MBC		37.5	18.8	18.8	>150	>150	>150	>150
Ginger powder (mg ml ⁻¹)	MIC	SDW	>150	>150	>150	>150	>150	>150	>150
	MBC		>150	>150	>150	>150	>150	>150	>150
	MIC	20% DMSO	75	150	>150	>150	>150	>150	>150
	MBC		150	150	>150	>150	>150	>150	>150
Cinnamon powder (mg ml ⁻¹)	MIC	SDW	37.5	150	75	75	75	75	75
	MBC		>150	>150	75	>150	>150	>150	>150
	MIC	20% DMSO	37.5	150	75	75	75	75	75
	MBC		150	150	>150	75	75	150	75
Turmeric powder (mg ml ⁻¹)	MIC	SDW	>150	>150	>150	>150	150	>150	>150
	MBC		>150	>150	>150	>150	>150	>150	>150
	MIC	20% DMSO	>150	>150	>150	>150	>150	>150	>150
	MBC		>150	>150	>150	>150	>150	>150	>150
Vancomycin (µg ml ⁻¹)	MIC	SDW	ND	ND	ND	ND	ND	ND	0.5
	MBC								>16

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SDW, sterile distilled water; DMSO, dimethyl sulfoxide; ND, not done.

Table 4 Susceptibility of micro-organisms to processed natural products by broth microdilution (20% DMSO was used as the primary solvent)

Processed products	Micro-organisms							
		<i>Staphylococcus aureus</i> ATCC 29213	<i>Escherichia coli</i> ATCC 25922	<i>Clostridium perfringens</i> ATCC 13124	<i>Clostridium difficile</i> ATCC 13366	<i>C. difficile</i> ribotype UK 014	<i>C. difficile</i> ATCC 43598	<i>C. difficile</i> ATCC 700057
Garlic tablet (mg ml ⁻¹)	MIC	37.5	37.5	75	37.5	37.5	75	37.5
	MBC	150	150	150	>150	>150	>150	>150
Ginger tablet (mg ml ⁻¹)	MIC	>150	>150	>150	>150	>150	>150	>150
	MBC	>150	>150	>150	>150	>150	>150	>150
Turmeric tablet (mg ml ⁻¹)	MIC	>150	>150	>150	>150	>150	>150	>150
	MBC	>150	>150	>150	>150	>150	>150	>150
Cinnamon tablet (mg ml ⁻¹)	MIC	>150	>150	>150	>150	>150	>150	>150
	MBC	>150	>150	>150	>150	>150	>150	>150
Artichoke tablet (mg ml ⁻¹)	MIC	150	150	150	75	75	150	75
	MBC	150	150	150	>150	>150	>150	>150
Coconut oil capsule (% v/v)	MIC	>32	>32	>32	>32	>32	>32	>32
	MBC	>32	>32	>32	>32	>32	>32	>32
Peppermint oil (% v/v)	MIC	8	16	8	8	8	8	8
	MBC	16	32	8	8	8	8	8
Aloe Vera gel (% w/v)	MIC	16	16	16	16	16	16	16
	MBC	32	32	>32	>32	>32	>32	>32
Allicin (mg ml ⁻¹)	MIC	18.8	18.8	4.7	4.7	2.3	4.7	4.7
	MBC	>37.5	37.5	4.7	>37.5	>37.5	>37.5	>37.5
Trans-cinnamaldehyde (% v/v)	MIC	0.02	0.06	0.01	0.02	0.02	0.02	0.02
	MBC	0.06	0.25	0.01	0.02	0.02	0.02	0.02
Zingerone (mg ml ⁻¹)	MIC	9.4	9.4	9.4	9.4	9.4	9.4	9.4
	MBC	37.5	9.4	18.8	9.4	18.8	9.4	9.4
Menthol (mg ml ⁻¹)	MIC	9.4	9.4	18.8	9.4	9.4	9.4	9.4
	MBC	37.5	9.4	18.8	18.8	18.8	18.8	18.8

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; DMSO, dimethyl sulfoxide.

flora of the gastrointestinal tract (Tables 5 and 6). It showed that lactic acid bacteria such as *L. casei* were less susceptible to most of the natural products used in this study compared to *Bacteroides* and *C. difficile*. There was at least a fourfold higher MIC observed for most active natural products against the two *L. casei* strains compared to the three *Bacteroides* species. Moreover, most agents did not show any bactericidal activity against lactic acid bacteria except peppermint oil and the four pure compounds.

The drug interaction assay using the checkerboard method showed that combining *trans*-cinnamaldehyde with vancomycin or metronidazole decreased the MIC values for both tested *C. difficile* isolates in comparison with either agent alone (Fig. 1). When *trans*-cinnamaldehyde was combined with vancomycin, there was at least a fourfold increase in inhibitory effect of vancomycin and a twofold rise in inhibitory activity of *trans*-cinnamaldehyde with most concentrations. All the FIC indices were <0.5 when tested against *C. difficile* ATCC 700057 and they ranged from 0.3750 to 0.5625 against *C. difficile* ribotype UK 027 (Table 7). Thus, *trans*-cinnamaldehyde enhanced the *in vitro* activity of vancomycin against the

two reference strains of *C. difficile* and based on the calculated FIC indices, *trans*-cinnamaldehyde had a possible synergistic effect with vancomycin. Moreover, the combination of *trans*-cinnamaldehyde and metronidazole resulted in at least twofold decrease in the MIC values of *trans*-cinnamaldehyde and metronidazole against *C. difficile* strains with FIC indices ranging from 0.5 to 0.625 (Table 7). Hence, a partial synergistic effect of *trans*-cinnamaldehyde with metronidazole might be considered. On the other hand, the combination of other natural agents with either vancomycin or metronidazole did not affect the MIC values relative to each therapeutic agent alone. Therefore, indifferent results were observed in combination assay for the remaining natural products against *C. difficile* isolates.

Discussion

Antibiotic treatment failure and increasing frequency of hypervirulent *C. difficile* strains have driven researchers to look for new therapeutic options for CDI, including complementary and alternative therapies. In this study, we investigated various natural products against *C. difficile*

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Table 5 Susceptibility of representative commensal flora of the gastrointestinal tract to raw natural products

			Micro-organisms				
Raw products		Solvent	<i>Bacteroides fragilis</i> NCTC 9343	<i>B. vulgatus</i> NCTC 10583	<i>B. thetaiotaomicron</i> 5018	<i>Lactobacillus casei</i> Q328	<i>L. casei</i> M2971
Garlic juice (% v/v)	MIC	SDW	1.6	0.8	0.8	6.3	6.3
	MBC		3.1	1.6	3.1	>50	>50
Ginger juice (% v/v)	MIC	SDW	>50	50	50	>50	>50
	MBC		>50	>50	>50	>50	>50
Onion juice (% v/v)	MIC	SDW	>50	50	>50	>50	>50
	MBC		>50	>50	>50	>50	>50
WA Manuka honey (% w/v)	MIC	SDW	2	1	1	32	32
	MBC		8	1	1	>32	>32
Garlic powder (mg ml ⁻¹)	MIC	SDW	9.4	9.4	9.4	37.5	37.5
	MBC		18.8	18.8	18.8	>150	>150
	MIC	20% DMSO	9.4	9.4	9.4	37.5	37.5
	MBC		18.8	18.8	18.8	>150	>150
Ginger powder (mg ml ⁻¹)	MIC	20% DMSO	150	150	150	>150	>150
	MBC		>150	150	>150	>150	>150
Cinnamon powder (mg ml ⁻¹)	MIC	20% DMSO	75	75	75	150	150
	MBC		150	75	150	>150	>150
Turmeric powder (mg ml ⁻¹)	MIC	20% DMSO	150	150	150	>150	>150
	MBC		>150	150	>150	>150	>150

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

Table 6 Susceptibility of representative commensal flora of the gastrointestinal tract to processed natural products (20% DMSO was used as a primary solvent)

Processed products		Micro-organisms				
		<i>Bacteroides fragilis</i> NCTC 9343	<i>B. vulgatus</i> NCTC 10583	<i>B. thetaiotaomicron</i> 5018	<i>Lactobacillus casei</i> Q328	<i>L. casei</i> M2971
Garlic tablet (mg ml ⁻¹)	MIC	75	75	75	150	150
	MBC	150	150	150	>150	>150
Ginger tablet (mg ml ⁻¹)	MIC	>150	>150	>150	>150	>150
	MBC	>150	>150	>150	>150	>150
Turmeric tablet (mg ml ⁻¹)	MIC	>150	>150	>150	>150	>150
	MBC	>150	>150	>150	>150	>150
Cinnamon tablet (mg ml ⁻¹)	MIC	>150	150	>150	>150	>150
	MBC	>150	>150	>150	>150	>150
Artichoke tablet (mg ml ⁻¹)	MIC	75	75	75	>150	>150
	MBC	150	150	150	>150	>150
Coconut oil capsule (% v/v)	MIC	>32	>32	>32	>32	>32
	MBC	>32	>32	>32	>32	>32
Peppermint oil (% v/v)	MIC	1	4	1	4	8
	MBC	1	4	1	8	8
Aloe Vera gel (% w/v)	MIC	16	16	32	32	32
	MBC	32	16	32	>32	>32
Allicin (mg ml ⁻¹)	MIC	9.4	9.4	9.4	37.5	37.5
	MBC	18.8	18.8	9.4	>37.5	>37.5
Trans-cinnamaldehyde (% v/v)	MIC	0.02	0.02	0.02	1	2
	MBC	0.1	0.03	0.03	2	2
Zingerone (mg ml ⁻¹)	MIC	4.7	4.7	4.7	18.8	18.8
	MBC	18.8	9.4	9.4	18.8	37.5
Menthol (mg ml ⁻¹)	MIC	4.7	4.7	4.7	18.8	18.8
	MBC	18.8	9.4	18.8	18.8	37.5

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

Figure 1 Isobolograms representing the effect of *trans*-cinnamaldehyde on antibiotics against *Clostridium difficile*. (a) *Trans*-cinnamaldehyde and vancomycin against *C. difficile* ATCC 700057, (b) *trans*-cinnamaldehyde and vancomycin against *C. difficile* UK 027 ATCC 13366, (c) *trans*-cinnamaldehyde and metronidazole against *C. difficile* ATCC 700057, (d) *trans*-cinnamaldehyde and metronidazole against *C. difficile* UK 027 ATCC 13366. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 7 MIC and FIC index values of *trans*-cinnamaldehyde and antibiotics against *Clostridium difficile* strains

Organism	Metronidazole			Vancomycin		
	Trans-cinn	MET	FIC	Trans-cinn	VAN	FIC
<i>C. difficile</i> ATCC 700057						
<i>Trans</i> -cinnamaldehyde	0.0313	–	–	0.0313	–	–
Antibiotic alone	–	0.5000	–	–	2.0000	–
Combination 1	0.0039	0.250	0.6250	0.0009	0.5000	0.2813
Combination 2	0.0078	0.1250	0.5000	0.0078	0.2500	0.3750
Combination 3	0.0156	0.0313	0.5624	0.0156	0.2500	0.3750
<i>C. difficile</i> ATCC 13366						
<i>Trans</i> -cinnamaldehyde	0.0313	–	–	0.0156	–	–
Antibiotic alone	–	4.0000	–	–	1.0000	–
Combination 1	0.0039	2.0000	0.6250	0.0019	0.250	0.3750
Combination 2	0.0078	1.0000	0.5000	0.0039	0.1250	0.3750
Combination 3	0.0156	0.5000	0.6250	0.0078	0.0625	0.5625

MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration; Trans-cinn, *trans*-cinnamaldehyde; MET, metronidazole; VAN, vancomycin.

and garlic was one of the products that showed that garlic was one product that demonstrated substantial activity against *C. difficile* isolates. Garlic has a broad spectrum of activity against a wide range of Gram-positive and Gram-

negative bacteria (Booyens and Thantsha 2013). Allicin is the main active compound in garlic that is responsible for its antibacterial activity (Booyens and Thantsha 2013; Sihombing *et al.* 2014). Other compounds in garlic such

as saponins and flavonoids may also contribute to its antibacterial activity (Booyens and Thantsha 2013). Alliin is generated by crushing garlic cloves, which results in the conversion of alliin to allicin and other thiosulfates by alliinase enzyme (Sihombing *et al.* 2014). As shown by the agar well diffusion assay, the highest activity was observed from garlic juice extracted from fresh garlic cloves. Moreover, since all garlic products showed good solubility in both SDW and 20% DMSO, this makes it a suitable and feasible agent to investigate. Agar well diffusion is a simple and quick method to generate and interpret the results (Hammer *et al.* 1999; Valgas *et al.* 2007). However, factors other than the susceptibility of the micro-organism may affect the size of the inhibition zone, such as the ability of products to diffuse in the medium, composition of medium and incubation conditions (Valgas *et al.* 2007; Boorn *et al.* 2010). This method may not be as sensitive as some other antimicrobial activity assays but it is used as a quick screening technique by many researchers and allows the comparison of results with those obtained by other methods (Boorn *et al.* 2010). The broth microdilution technique provides more quantitative results than agar diffusion, allowing to distinguish inhibitory and bactericidal activity. Even though this technique is applied extensively, limitations still exist such as poor growth of anaerobic species. However, this study followed the methods recommended by CLSI, thus, more consistent and reliable results were generated.

Very few studies have investigated the antibacterial effect of natural products on *C. difficile*, thus limiting comparison of results. In a study by Hammond and Donkor (2013), the effect of Woundcare™ 18+ Active manuka honey from Comvita UK against three *C. difficile* isolates was investigated using both agar well diffusion and broth microdilution techniques. In their study, 50% manuka honey solution showed zones of inhibition ranging from 14.2 to 14.5 mm after 2 days of incubation for the three strains of *C. difficile* which included a reference strain (ATCC 9689) and two clinical isolates of PCR ribotypes 027 and 106. In addition, MIC and MBC values of 6.25% v/v were observed for manuka honey against the three *C. difficile* isolates, suggesting bactericidal activity (Hammond and Donkor 2013). In our study, MICs for the WA-produced manuka honey were more than fourfold higher against the four *C. difficile* isolates than those obtained in Hammond and Donkor (2013) study and no bactericidal activity was observed at the highest concentration of honey solutions used. This could be because different strains of *C. difficile* were used in each study, however, is also likely due to differences between the two honey samples tested, as the activity of honey can vary significantly due to the spatial and temporal variation in sources of nectar (Kwakman *et al.* 2008).

Another study investigated the effect of virgin coconut oil on toxin A- and toxin B-positive *C. difficile* (ATCC 9689) *in vitro* (Shilling *et al.* 2013). Virgin coconut oil did not show any inhibitory effect against this *C. difficile* strain, however, when the coconut oil was digested with porcine lipase, the resulting product inhibited 99.9% of overnight growth of *C. difficile* at concentration of 1.2% v/v (Shilling *et al.* 2013). These findings correlated with the results from our study, as no antimicrobial activity was observed for undigested virgin coconut oil against any of tested micro-organisms using either agar well diffusion or broth microdilution techniques.

Both vancomycin and metronidazole possess antibacterial activity against *C. difficile*, however, there is a risk of infection recurrence following treatment (Rea *et al.* 2011). Antibiotics used for therapy may enhance the risk of recurrent infection by having a negative impact on the gut microflora (Rea *et al.* 2011). The impact of antibiotics such as vancomycin and metronidazole on intestinal flora has been studied previously (Edlund *et al.* 1997; Al-Nassir *et al.* 2008; Rea *et al.* 2011). For example, Edlund *et al.* (1997) assessed the effect of oral vancomycin on the intestinal flora of patients who had previously received antimicrobial therapy (Edlund *et al.* 1997). They showed that bifidobacteria and *Bacteroides* species were eliminated during vancomycin administration and this resulted in the emergence of vancomycin-resistant enterococci, *Pedococcus* species, and lactobacilli (Edlund *et al.* 1997; Rea *et al.* 2011). In our study, the selected natural products had less impact on *L. casei in vitro* and both *L. casei* strains were less susceptible to most of the natural therapeutic agents compared to *C. difficile* and *Bacteroides*. This is a potential advantage of using natural products in that they have activity against *C. difficile* at concentrations lower than those that affect *Lactobacillus* species. Thus, they may be considered as a supplementary therapeutic option that is less disruptive to the commensal flora. *Lactobacillus casei* is a commercial probiotic strain and use of anti-*C. difficile* agents with less potential activity against this commensal organism is beneficial for treatment. A previous study has suggested the benefit of using garlic as a health supplement against various pathogens including clostridia in the gut, as it would not adversely impact the commensal flora of the gastrointestinal tract (Filocamo *et al.* 2012). In that study, *L. casei* was also tested and unlike the rapid killing of *Bacteroides* and bifidobacteria by garlic, *L. casei* strains showed less susceptibility (Filocamo *et al.* 2012).

Vancomycin is a glycopeptide antibiotic that can inhibit cell wall synthesis in susceptible micro-organisms by preventing the polymerization of phosphodisaccharide-penta-peptide lipid complex (Watanakunakorn 1984). A few studies have shown other possible additional modes

of action for vancomycin such as alteration of cell membrane and selectively inhibiting ribonucleic acid synthesis (Hancock and Fitz-James 1964; Watanakunakorn 1984). On the other hand, metronidazole acts on susceptible micro-organisms by inhibiting DNA synthesis and breaking DNA strands by oxidation that leads to cell death (Löfmark *et al.* 2010). *Trans*-cinnamaldehyde is an essential oil component of cinnamon. It has been postulated that the antimicrobial activity of essential oil components is mostly due to their hydrophobic nature that allows them to penetrate microbial membranes, alter their integrity which leads to the leakage of cell constituents (Nazzaro *et al.* 2013). The cell wall structure of Gram-positive bacteria allows hydrophobic molecules to penetrate the cell and affect both cell wall and cytoplasmic content. In contrast, the outer membrane of Gram-negative bacteria does not allow the penetration of hydrophobic molecules as readily in comparison to Gram-positive bacteria (Nazzaro *et al.* 2013). The outer membrane of Gram-negative bacteria is not completely impermeable to hydrophobic molecules with only some being able to pass through porins in the outer membrane (Wendakoon and Sakaguchi 1995). Essential oils and their components can have more than one target for their activity. For example, *trans*-cinnamaldehyde can prevent the growth of *E. coli* without altering the outer membrane of the bacterial cell but is likely to gain access to the periplasm and deeper portions in bacterial cell (Helander *et al.* 1998). In a study by Domadia *et al.* (2007) it was shown that *trans*-cinnamaldehyde inhibited cell division in *Bacillus cereus* (Domadia *et al.* 2007). Different mechanisms of action have been proposed for *trans*-cinnamaldehyde including the inhibition of cell wall synthesis similar to vancomycin, which would explain how *trans*-cinnamaldehyde may enhance the activity of this antibiotic. In addition, if inhibition of cell division is another possible mechanism of action for *trans*-cinnamaldehyde it would clarify the partial enhancement of activity observed with *trans*-cinnamaldehyde and metronidazole, as this antibiotic acts by inhibiting DNA synthesis. The remaining natural agents did not reduce the effect of either antibiotic, therefore the consumption of those natural products that had activity against *C. difficile* in conjunction with antibiotics may be considered. However, some of these products such as *trans*-cinnamaldehyde are toxic to humans if consumed in high doses and precautions regarding the safe use of these products should be followed. Many natural products including garlic have generally recognized as safe status, which means when consumed in moderate amounts are unlikely to pose a health risk (U.S. Department of Health and Human services, 2015).

Natural products should not always be assumed to be safe as side effects are expected with some (World Health

Organisation 2004). Some of the adverse events that may arise from the use of medicinal plants are due to the use of the wrong species of plants, incorrect dosing, interactions with other medications and contamination with hazardous substances (Nasri and Shirzad 2013). The safety of herbal medicines is important for the public health, and therefore it is necessary to identify the risks associated with their use through preclinical and clinical toxicological evaluation of medicinal plants (Moreira *et al.* 2014). This enables identification of toxic compounds which can be discarded or modified to provide a safer alternative (World Health Organisation 2004). Moreover, dosage adjustment and modification of certain chemical structure may increase their tolerability.

In conclusion, this study highlights the antimicrobial activity of a number of natural products against *C. difficile* *in vitro* while having less effect against commensal flora in the gastrointestinal tract such as *L. casei*. This suggests that the active natural products may be potential alternative treatment options for CDI. Furthermore, as the low concentrations of *trans*-cinnamaldehyde enhanced the antimicrobial activity of conventional antibiotics *in vitro*, the combination therapy of *trans*-cinnamaldehyde with antibiotics may enhance the treatment outcome for this infection. Further studies exploring the effect of this treatment on the virulence factors produced by *C. difficile* and their activity *in vivo* are worth doing. Lastly, studies investigating the effects of natural products on *C. difficile* endospore production, germination and outgrowth are currently underway.

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Conflict of Interest

No conflict of interest is declared.

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Natural products for the treatment of *Clostridium difficile* infection

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Review article

C.difficile (including epidemiology)

Non-conventional antimicrobial and alternative therapies for the treatment of *Clostridium difficile* infection

Niloufar Roshan^a, Katherine A. Hammer^a, Thomas V. Riley^{a, b, c, d, *}

^a School of Biomedical Sciences (M504), The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia

^b Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA 6009, Australia

^c School of Veterinary & Life Sciences, Murdoch University, Murdoch, WA, Australia

^d School of Medical & Health Sciences, Edith Cowan University, Joondalup, WA, Australia

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ABSTRACT

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus that causes disease ranging from self-limiting diarrhoea to severe pseudomembranous colitis. *C. difficile* infection (CDI) commonly affects hospitalised patients and is increasingly identified in patients in the community with no hospital contact. For the last 15 years the incidence of CDI worldwide has been rising, especially in the northern hemisphere. The yearly average number of hospitalizations as a result of this disease is estimated to be over a quarter of a million per year in the United States alone. The main risk factor for CDI is exposure to antimicrobials that affect the gut microflora and, paradoxically, the most common treatments for CDI are the antimicrobials, metronidazole and vancomycin. However, the increasing frequency of highly virulent *C. difficile* strains, antimicrobial treatment failures, hospital outbreaks, patients with severe complications and cases with multiple recurrences have driven the search for new therapies.

Several novel or popular complementary and alternative therapies are self-prescribed for treatment of other diarrheal diseases, and these may also be appropriate for treating CDI. In general, complementary and alternative medicine (CAM) is mainly used by patients when conventional therapeutic agents show limited success against *C. difficile* and other antimicrobial-resistant bacteria. Among these alternative approaches, a number of treatments, such as herbal remedies, are embraced less by pharmaceutical and medical professions. This review summarises current knowledge of non-conventional antimicrobial and alternative therapies for treatment of CDI. As the demand for non-conventional antimicrobial therapies increases, further studies are required in the field of CAM, especially natural products, for the treatment of CDI.

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* Corresponding author. Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA 6009, Australia.

E-mail address: thomas.riley@uwa.edu.au (T.V. Riley).

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1. Introduction

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus and clinical signs associated with *C. difficile* infection (CDI) can range from mild diarrhoea to fulminant colitis [1]. Conventional treatment of CDI relies on two commonly used antimicrobials, metronidazole and vancomycin, however, they also deplete gut flora, neither is effective against *C. difficile* spores and both result in a significant rate of recurrence of disease [2]. Fidaxomicin is a new macrocyclic drug suggested for use in severe cases of CDI, when the patient is not responding to vancomycin. It has a narrower spectrum of activity compared to vancomycin and metronidazole, and thus may have less impact on gut microbiota [2].

While the most common manifestation of CDI is diarrhoea, this can also occur in a range of other gastrointestinal disorders [1]. There are various studies examining the use of complementary and alternative medicine (CAM) in the treatment of such gastrointestinal disorders. The term CAM encompasses a wide range of practices including but not limited to herbal remedies, nutritional supplements and meditation [3]. CAMs may be used by people as a personal luxury item (for no particular health reason), as a preservative (to boost health) or as a treatment option [4]. In this review, the different treatment options for CDI are described. Furthermore, how alternative therapeutic agents that are used primarily for treating other diarrhoeal-associated diseases may be considered as appropriate for CDI is also explored.

2. *Clostridium difficile*

2.1. Risk factors

The intestinal microbiota play a critical role in human health and their disruption increases the susceptibility of the host to CDI [5]. Commensal flora prevent the overgrowth of enteric pathogens such as *C. difficile* by competing for nutrients, production of bacteriocins and contributing to host responses.

Exposure to antimicrobials remains the major predisposing factor that increases the risk of contracting CDI by disrupting the gut microbiota. Prolonged hospitalization, or residency in nursing homes and rehabilitation centres, also are associated with greater risk [5]. Another known risk factor is advanced age, as people over 65 years of age are more likely to contract *C. difficile*. In addition, immunosuppression and use of gastric acid suppressors increase the risk of developing CDI [5,6].

2.2. Pathogenesis of CDI

There are a number of virulence factors produced by *C. difficile*

that contribute to the pathogenesis of CDI. The major virulence factors that are involved in initiating disease are the glucosyl-transferase toxins TcdA and TcdB [7]. The corresponding toxin genes *tcdA* and *tcdB* are located on a 19.6-kb chromosomal region called the pathogenicity locus (PaLoc) [8]. There are three accessory genes located on the PaLoc including *tcdR* (activator), *tcdC* (negative regulator) and *tcdE* (a putative holin) [8]. Toxin A is described as an enterotoxin that causes exudative colitis by disrupting the adherence of colonic mucosal cells to the colonic basement membrane and destroying the villous tips. Toxin B has cytotoxic activity and enters the cell by endocytosis causing apoptosis and cell death [7,8]. There is an additional toxin (binary toxin) produced by a smaller proportion of *C. difficile* strains such as the so-called hyper-virulent BI/NAP1/027 epidemic strain. However, there are still gaps in our knowledge of this toxin and how it contributes to virulence [9].

2.3. Clinical presentation of CDI

The clinical presentation of CDI ranges from mild diarrhoea to fulminant colitis. The time between antimicrobial exposure and onset of symptoms may vary from 1 day to 6 weeks or in some cases even longer [10]. The severe forms of disease, such as fulminant colitis, are least common and usually occur in only 1%–3% of patients [11]. The prominent clinical features in mild to moderate disease include watery diarrhoea, mild abdominal pain and cramps [10,11]. In severe cases of CDI, the patient can suffer from additional symptoms such as fever, leukocytosis, hypoalbuminemia, severe lactic acidosis, diffuse abdominal pain and distention due to severe toxicity [11].

3. Treatment options

3.1. Conventional antimicrobial treatments

Metronidazole and vancomycin have been the mainstay of CDI treatment for many years. Currently, metronidazole is the antimicrobial of choice for mild to moderate cases of CDI [12]. Both vancomycin and metronidazole show similar cure rates in patients with initial or recurrent mild to moderate infection, however, vancomycin is reserved for severe cases due to growing concerns about the generation of antimicrobial-resistant organisms [2,13]. Unlike metronidazole, oral vancomycin is Food and Drug Administration (FDA) approved for treating CDI and is more expensive than metronidazole [14,15]. In general, based on previous randomized, controlled trials, the cure rates for mild CDI with metronidazole and vancomycin were 90% and 98%, respectively [16]. However, in severe disease, only 76% of patients taking metronidazole were cured, while 97% of those taking vancomycin were [16]. Therefore, despite the typical efficacy of vancomycin and

metronidazole, there is concern about treatment failures with both antimicrobials and the increasing recurrence rate.

Fidaxomicin is new FDA-approved antimicrobial which became available for the treatment of CDI in 2011. One of the advantages of fidaxomicin over vancomycin is that it is bactericidal, whereas vancomycin has only bacteriostatic activity against *C. difficile* [13]. In a phase III trial of fidaxomicin, the rates of cure and recurrence in patients with the BI/NAP1/027 strain of *C. difficile* were similar to those taking vancomycin [17]. For 64% of patients with strain types other than 027, patients treated with fidaxomicin had a recurrence rate of 7.85% compared to a 25.25% recurrence rate in those treated with vancomycin [17]. However, the cost of this antimicrobial for a 10-day course of treatment is 125-fold higher than either metronidazole or vancomycin for the same duration [17] and, therefore, the high cost of fidaxomicin may restrict its use.

3.2. Alternative antimicrobials

Teicoplanin is a glycopeptide antimicrobial with activity against Gram-positive anaerobes such as *C. difficile* [1,14]. A randomized, controlled clinical trial published in 1996 comparing the activity of the four different antimicrobials metronidazole, vancomycin, teicoplanin and fusidic acid showed that teicoplanin was as effective as vancomycin and metronidazole for CDI [14]. However, there was a high cost associated with teicoplanin use that made it a last line drug reserved for the treatment of repeated recurrences of CDI.

Tigecycline is a broad-spectrum antibiotic with *in vitro* activity against various Gram-positive and Gram-negative bacteria as well as several anaerobic microorganisms [12,14]. *In vitro* studies have shown a MIC₉₀ ranging from 0.016 to 0.25 mg ml⁻¹ for all *C. difficile* isolates. Treatment success was reported in six cases of CDI that were refractory to metronidazole and vancomycin [12]. However, inhibition of gut microbiota has been observed with this antibiotic and more studies are required to assess the safety and efficacy of tigecycline in treatment of CDI [12].

A number of novel antimicrobial agents such as ramoplanin, surotomycin, cadazolid and ridinilazole are currently being investigated in clinical trials for treatment of CDI [1,18–20]. Ramoplanin is a lipoglycopeptide with *in vitro* activity against many aerobic and anaerobic bacteria [18,20]. In a phase II trial, the efficacy of ramoplanin was compared to vancomycin in 89 patients with CDI. Ramoplanin at doses of 200 or 400 mg administered twice daily for 10 days showed equivalent efficacy to vancomycin. However, there was not sufficient power in this study to demonstrate non-inferiority of ramoplanin [20].

Surotomycin is an orally dosed lipopeptide antibiotic that acts by disrupting the cell membrane [19]. In a randomized, phase II trial, the response rates with either doses of surotomycin (125 and 250 mg) were similar to that of vancomycin (92 and 87 versus 89%, respectively) [20]. The recurrence rate was lower with surotomycin compared to vancomycin (17.2 and 27.9 versus 35.6, respectively), however, the trial was too small to show a statistically significant difference. Moreover, the data from the phase III trials did not demonstrate non-inferiority of surotomycin to vancomycin [20].

Another novel antimicrobial cadazolid is an oxazolidinone-fluoroquinolone that acts by inhibiting protein synthesis in *C. difficile* [19,20]. In a phase II trial, cadazolid had a better sustained cure rate compared with vancomycin (60, 56, and 47% for 250, 500 and 1000 mg cadazolid, respectively, and 33% for vancomycin) [19]. However, a low overall response rate was observed for vancomycin. A phase III trial assessing the safety and efficacy of cadazolid has been recently completed, but no study results has yet been reported (NCT01983683).

Ridinilazole (formerly known as SMT19969) is a non-absorbable, narrow spectrum antimicrobial with high activity

against *C. difficile in vitro* [19]. In a phase II randomized clinical trial, ridinilazole was non-inferior to vancomycin with sustained clinical response rates of 67 and 42%, respectively. The recurrence rate was also lower with ridinilazole compared to vancomycin (14.3 vs. 34.8%). However, vancomycin showed a lower efficacy in this trial compared to previous trials [19].

3.3. Conventional non-antimicrobial treatments

3.3.1. Faecal microbiota transplantation (FMT)

Faecal microbiota transplantation (FMT) is an effective method of treatment for recurrent CDI [21]. This procedure is usually recommended after a third recurrence of CDI and restores the normal composition of the intestinal flora by transferring faeces from a healthy donor to the patient, also stimulating the immune response in the recipient [21,22]. FMT is not approved as a treatment modality by the European Medicines Agency (EMA) nor the US FDA [23]. Several stool banks have been established around the world to overcome the hurdles associated with lack of availability of donor faeces and safety concerns. A stool bank needs to be in close proximity to a clinical microbiology department with experts in the field of microbiology and infectious diseases to screen and process the samples [23].

The first faecal transplant was performed in 1958 on four patients with pseudomembranous enterocolitis and this resolved disease in all four [24]. A subsequent study reported a success rate of 92% in patients with a history of multiple recurrent CDI after receiving a stool transplant [24]. Of a total of 26 patients who underwent the procedure, 24 remained free of any symptoms of CDI [24]. In a randomized, controlled trial by Van Nood et al. the efficacy of three treatment regimens was compared in 43 patients with recurrent CDI [25]. The patients were randomly assigned to receive infusion of donor faeces, vancomycin or vancomycin and bowel lavage [25]. Based on the results, 81% of patients in infusion group recovered after first infusion of donor faeces. Resolution of infection among the other two groups receiving either vancomycin alone or vancomycin and bowel lavage was 31% and 23% respectively. No major adverse effect was observed among the three groups [25].

While the success of faecal bacteriotherapy has been reported in several studies, the practical barriers and adverse events must be considered; for instance administration of stool transplant by colonoscopy or duodenal tubes causes discomfort in patients and exposes them to some risk [26]. In a recent cohort study by Youngster et al., the efficacy of orally administered frozen faecal capsules in 180 patients with recurrent CDI was assessed [27]. The study showed a cure rate of 91% with either one or two treatments, however, no placebo group was included [27]. Three adverse events including one patient with high fever and two with ulcerative colitis with possible relation to FMT were reported [27]. Oral administration of faecal flora is a simple and cost-effective technique for the treatment of recurrent CDI, but there are potential limitations; capsule preparation is labor-intensive, it requires patients' cooperation and its use is limited in patients with dysphasia. Furthermore, despite the benefits associated with faecal bacteriotherapy, there remains uncertainty about its long-term safety [26].

3.3.2. Immunotherapy

Since *C. difficile* toxins A and B are the main factors responsible for the symptoms observed in *C. difficile*-associated diseases, they can be considered as prime targets for effective protection or treatment. Kyne et al. investigated the association between the antibody responses against *C. difficile* in 63 patients with recurrent *C. difficile* associated diarrhoea (CDAD) [28]. Based on their findings, 50% of 44 survivors of CDAD experienced recurrence of the disease.

A higher serum IgG response to toxin A was detected in patients with a single episode of disease compared to patients who had subsequent recurrent diarrhoea [28], illustrating the importance of an adequate immune response in providing protection against recurrence. In two randomized controlled trials with 2655 patients with CDI who were receiving antibiotic treatment, the efficacy of the monoclonal antibodies bezlotoxumab (anti-toxin B) alone or in combination with actoxumab (anti-toxin A) for prevention of recurrent CDI was investigated [29]. Bezlotoxumab therapy resulted in a lower rate of recurrent CDI [29]. Despite the success of this monoclonal antibody in treatment of CDI, there remain concerns over the costs associated with antibody production.

Active vaccination to prevent CDI has been a long-standing goal of *C. difficile* researchers. A study published in 2011 showed that oral immunisation with *Bacillus* spores expressing toxin A peptide repeats induced protection against lethal doses of *C. difficile* producing toxin A and B in a hamster model of CDI [30]. This vaccination strategy was based on induction of secretory IgA with cross-reactivity against toxin B which was also heat stable [30].

Several studies have also investigated the efficacy of recombinant vaccines in animal models of CDI [31–36]. A recent study investigated the development of a tetravalent vaccine against CDI containing recombinant, attenuated TcdA, TcdB and binary toxin components CDTa and CDTb. The study was done using the Syrian golden hamster model and showed that inclusion of binary toxin components significantly increased the survival rate in hamsters challenged with BI/NAP1/027 strains compared to bivalent vaccines containing only TcdA and TcdB antigens [36].

Despite successful studies on vaccine efficacy, there are manufacturing challenges involved with production of a toxoid vaccine such as safe handling of toxin, decontamination of spores and adjusting the amount of each of the toxins produced from the same strain. Sanofi has recently announced a decision to discontinue clinical development of its experimental toxoid vaccine. According to the company statement, following a planned interim analysis of a phase III clinical trial, it was concluded that the probability that the study will meet the primary objective was low [37].

3.4. Complementary and alternative therapies

Increasing incidence and recurrence rates of CDI have been observed since the early 2000s [18]. Also, there has been a growing failure rate of existing antimicrobial therapies in treating *C. difficile* which highlights the need for development of alternative therapeutic approaches. The term CAM encompasses a wide range of practices including but not limited to herbal remedies, nutritional supplements and meditation [38]. A variety of non-conventional therapies for the treatment of CDI have been investigated, most of which are categorised as antimicrobial agents, toxin binding agents, probiotics or bacteriocins (Table 1).

3.4.1. Toxin binding agents

C. difficile initiates disease by releasing toxins in the colonic lumen, however, it is not usually invasive [7,38]. This has prompted researchers to investigate compounds that may bind to the toxins before their attachment to the enterocytes. There are several types of adsorbents that have been investigated in the treatment of CDI. These include ion exchange resins such as cholestyramine and colestipol, polymers (e.g. tolevamer) and oligosaccharides (e.g. Synsorb 90) [38].

Previous studies on colestipol and cholestyramine showed that cholestyramine is more effective at binding *C. difficile* toxins than colestipol *in vitro* [39]. They were both initially used as adjunctive therapies with vancomycin. Later, using hamster models,

vancomycin was more effective than cholestyramine as a sole therapy. However, both colestipol and cholestyramine can interfere with vancomycin efficacy if co-administered [39]. Therefore, vancomycin needs to be administered separately or to be taken hours apart from ion exchange resins to prevent interference in therapeutic efficacy.

Tolevamer is a toxin binding polymer which binds to *C. difficile* toxin in the intestine and, unlike ion exchange resins, does not bind to metronidazole or vancomycin [1]. Phase I and II trials testing tolevamer for the treatment of *C. difficile* showed its safety and efficacy for mild to moderate CDI [1]. However, in a phase III trial it was inferior to vancomycin and found not to be ideal for reducing levels of *C. difficile* toxin [1]. Furthermore, initial studies with oligosaccharides such as Synsorb 90 also showed encouraging results and in a phase II trial reduced recurrence rates of CDI [40]. Nevertheless, after a phase III trial, this drug was discontinued also and no further data have been published [40].

3.4.2. Bacteriophages

Research on use of bacteriophages or phage therapy as a therapeutic option for infectious diseases began soon after their discovery in 1915. Several phages have been identified and studied that infect *C. difficile* and they all contain elements with lysogeny control such as repressors, anti-repressors and integrases [41,42]. In a study by Meader et al. the effect of two different phage treatment regimes, prophylactic and remedial, in prevention and treatment of *C. difficile* *in vitro* was investigated [41]. A specific bacteriophage (ΦCD27) induced from *C. difficile* NCTC 12712 was used in this study. The remedial treatment showed a significant decrease in *C. difficile* count ($P < .0001$) and reduced toxin production by *C. difficile*. The prophylactic treatment prevented *C. difficile* growth ($P < .001$) and inhibited toxin production. Also none of the phage treatment regimes affected the gut commensal flora significantly [41]. Another study demonstrated phage combinations reducing *C. difficile* growth *in vitro* and proliferation *in vivo* [43]. Seven phages including six myoviruses and one siphovirus were used in this study in which the single phage treatment showed an initial reduction of bacterial load, followed by the emergence of phage-resistant colonies. On the other hand, use of specific phage combinations resulted in complete lysis of *C. difficile* *in vitro*. In a hamster model, oral administration of optimized phage combinations reduced *C. difficile* colonization [43].

3.4.3. Probiotics

Probiotics are live microorganisms that when administered to the host, enhance the indigenous microflora and re-establish the normal flora after disruption due to antimicrobial administration or other factors [44]. Probiotic organisms also stimulate the immune response and the production of antimicrobial molecules that degrade bacterial toxins or block their adhesion sites. The most common microorganisms utilized as probiotics for *C. difficile* are *Lactobacillus* species, *Saccharomyces boulardii* and bifidobacteria [44].

The severity of CDI is usually influenced by the immune response to *C. difficile* toxins. For example, high titres of anti-toxin A antibodies reduce the severity of CDI. *S. boulardii* is a yeast that can upregulate the expression of anti-toxin A secretory IgA and secrete a protease that degrades toxin A and B produced by *C. difficile* [44]. In a double-blind placebo-controlled trial, high doses of vancomycin in combination with *S. boulardii* significantly reduced the rate of recurrence of CDI compared to patients receiving high dose vancomycin and placebo [38].

Probiotics are also used in the treatment of other gastrointestinal disorders. For instance, several studies have shown the effectiveness of probiotics in the treatment of inflammatory bowel

Table 1
Non-conventional complementary and alternative approaches for treatment of *C. difficile* infection.

Agents	<i>In vitro</i> studies	Pre-clinical studies	Clinical studies
Toxin binding agents			
Colestipol/ cholestyramine	Toxin binding [39]	Therapeutic efficacy in a hamster model of clindamycin induced cecitis [39]	Several case reports of cholestyramine in treatment CDI relapses [18] Randomized controlled trial of colestipol in 37 patients with severe antimicrobial-associated diarrhoea [77]
Tolevamer	Toxin binding of Tolevamer [1,78]	Effect of tolevamer in treatment of hamsters with <i>C. difficile</i> colitis [78] Toxin neutralizing ability of tolevamer in a rat ileal loop model [78]	Multicentre, 3-arm, randomized, double-blind controlled phase II study on 289 patient with CDAD [79] Two phase III clinical trials involving more than 1100 patients with CDI [80]
Synsorb 90	Toxin neutralizing activity [40]	Effect of synsorb on toxin A-induced enteritis in rat ileum [40,81]	Phase II clinical trial on 103 patients with at least one CDI recurrence [40] Phase III trial was begun, but no data was ever published [40]
Bacteriophage			
	Morphological and genetic analyses of temperate phages in <i>C. difficile</i> [82] Characterizing temperate bacteriophages in <i>C. difficile</i> O27 strains [83] The efficacy of two phage treatments in prevention or treatment of CDI <i>in vitro</i> [41] Use of bacteriophage ΦCD27 in a human colon model system <i>in vitro</i> [43] The ability of phage combinations in effective clearance of <i>C. difficile</i> [84]	The potential application of phage combinations in eradication of CDI in a hamster model of acute CDI [84]	A pilot observational study investigating the bacteriophage transfer in 24 patients with CDI during FMT [85]
Probiotics			
	—	—	Double-blind, randomized, placebo-controlled study of <i>Saccharomyces boulardii</i> on 124 patients with <i>C. difficile</i> associated disease [80] Randomized double-blind placebo-controlled study of <i>S. boulardii</i> on 168 patients with recurrent CDI [86] Prospective, placebo-controlled multicenter trial of <i>Lactobacillus plantarum</i> 299v in 21 patients with recurrent CDI [87]
Bacteriocins			
	Antimicrobial activity of GE2270, nisin, lactacin 3147, Thuricin CD, actagardine, LFF571 and diffocins [50,51] Effect of nisin on spore outgrowth [88] Evaluating the activity of NVB302 for the treatment of CDI in an <i>in vitro</i> human gut model of CDI [50] Avidocin-CD killing activity [89]	Efficacy of LFF571 for recurrence of CDI in hamsters [50] Efficacy of Avidocin –CDs in Golden Syrian hamster model of acute CDI [89]	A randomized, double-blind, placebo-controlled study on 56 healthy male and non-pregnant female subjects between 18 and 65 years of age, assessing the safety and pharmacokinetics of single and multiple oral doses of LFF571 [90]
Natural products			
	Antimicrobial activity of Manuka honey [58], virgin coconut oil [59], pomegranate extract [60], black seed oil and Myrrh [61]	—	—

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disease (IBD) [45,46]. *Lactobacillus* GG administration has been successful in reducing the duration of diarrhoea in children with irritable bowel syndrome (IBS) [47]. In another study, patients in remission from ulcerative colitis who were allergic or intolerant to 5-aminosalicylic acid, an anti-inflammatory drug used in IBS, were given a mixture of probiotics, composed of three strains of bifidobacteria, four strains of lactobacilli and one strain of *Streptococcus salivarius* subsp. *thermophilus* [48]. No major side effects were observed in the patients and the probiotic organisms were able to colonise the intestine and improve the patients' recovery.

3.4.4. Bacteriocins of probiotics

Another treatment option for CDI is the administration of bacteriocins. Bacteriocins are ribosomally-synthesized peptides or proteins that are produced by different bacteria; for instance some probiotic organisms produce bacteriocins [49]. Several unique properties of bacteriocins make them viable alternatives to antimicrobials due to having either a narrow or broad spectrum of antimicrobial activity, low toxicity, the possibility of their production by probiotic organisms *in situ* and the ability to be bio-engineered [50]. For a bacteriocin to be considered as an alternative therapy for CDI it needs to be as effective as currently used antimicrobials and cause only minor damage to the gut flora. Previous studies have demonstrated *in vitro* activity of several bacteriocins against *C. difficile* [50,51]. Nisin and lactacin 3147 are two bacteriocins that are effective in killing *C. difficile* *in vitro* at concentrations that are comparable to vancomycin and metronidazole [50]. Another known bacteriocin, thuricin CD, is a two component peptide that has a narrower spectrum of activity making it a potential treatment for this infection [50,52].

GE2270 and its semi-synthetic derivative LFF571 are two other bacteriocins with antimicrobial activity against *C. difficile* [50]. A pre-clinical study has shown survival benefits of LFF571 compared to vancomycin at lower dose and with fewer recurrences in the hamster model of CDI [53]. Furthermore, in a randomized, double-blind, placebo-controlled human study, the safety, tolerability and pharmacokinetics of single and multiple ascending oral doses of LFF571 were investigated. Among 56 subjects, LFF571 was well-tolerated and no serious side effect was noted. A low concentration of LFF571 remained in serum and a high concentration was detected in faeces. The result supported the future development of LFF571 as a potential treatment for CDI [54].

3.4.5. Natural products

CAM rely mostly on traditional and natural remedies that are generally considered by many people to be less toxic than most conventional agents [55]. Medicinal plants have been used as a traditional treatment for various illnesses for thousands of years in many parts of the world [55]. In the rural areas of many developing countries, herbal therapies are still used as the primary source of treatment for many diseases. For instance, for over 5000 years, traditional Chinese medicine using various plant extracts has been the main treatment used by the Chinese population [56]. A study investigating the use of CAM in patients with gastrointestinal issues showed that 26% used CAM (mostly herbal/dietary therapy, acupuncture and mind/body based approaches) as a treatment option with nearly half being herbal therapy [57]. For the most part, various herbal remedies are used by patients to treat diarrhoeal diseases without having knowledge of the specific gastrointestinal problem or aetiology of the disease. These herbal remedies may have a role in treatment of CDI worth investigating.

A broad range of natural products has been evaluated for anti-*C. difficile* activity *in vitro*. In a study by Hammond and Donkor, the antimicrobial activity of manuka honey was investigated against three *C. difficile* strains [58]. The minimum inhibitory and minimum

bactericidal concentrations were 6.25% (v/v^{-1}) for the *C. difficile* strains. Since manuka honey showed a bactericidal effect against *C. difficile* *in vitro*, it suggests that this honey might be considered as an alternative treatment for CDI if future *in vivo* studies show potential [58]. Another study investigated the antimicrobial effect of virgin coconut oil against *C. difficile* [59]. This study showed that virgin coconut oil itself did not inhibit the growth of *C. difficile*, however lipolyzed virgin coconut oil at concentrations of 0.15%–1.25% v/v^{-1} inhibited *C. difficile* [59]. These results should encourage a more complete evaluation of virgin coconut oil and its bioactive components against *C. difficile*.

The antimicrobial activity of pomegranate extracts against 29 clinical *C. difficile* isolates has been investigated using an agar dilution method [60]. Also, the phenolic contents of pomegranate was determined using the Folin-Ciocalteu colorimetric method and final concentrations of 6.25–400 $\mu\text{g ml}^{-1}$ gallic acid equivalent were found. The MIC values for the extracts ranged from 12.5 to 25 mg ml^{-1} for all the strains, which suggested possible efficacy of pomegranate in treatment of CDI [60]. In another recent study, the antimicrobial activity of black seed oil and myrrh were investigated against two *C. difficile* strains using an agar well diffusion assay [61]. Both black seed oil (2% v/v^{-1}) and myrrh water extract inhibited *C. difficile* *in vitro* [61].

There are a number of commercially available supplements and preparations for the relief of symptoms experienced in several gastrointestinal disorders, including ginger, peppermint oil and aloe vera [62]. In particular, some essential oils have antispasmodic and carminative properties [62]. Peppermint oil has been the main constituent of many remedies for treating the symptoms of IBS. In a prospective, randomized, double-blind, placebo-controlled clinical study, 110 outpatients suffering from IBS were assigned to take either enteric-coated peppermint oil capsules or a placebo [63]. There was significant improvement of symptoms in patients taking the peppermint oil capsules compared to placebo. Ginger is another home remedy that is used for gastrointestinal symptoms. It has an anti-inflammatory effect and is thought to strengthen the gastric lining and trigger intestinal motility [64]. In a double blind randomized controlled pilot trial in 45 patients with IBS, ginger was well tolerated, but did not perform better than placebo in treating symptoms [64]. Nevertheless, ginger is a common herbal medicine used by IBS patients and larger trials are needed before drawing any definitive conclusions [64]. These commonly used natural products may be effective in the treatment of CDI.

Traditional Chinese herbal medicine has been used for centuries for the treatment of gastrointestinal disorders, especially for functional bowel disorders such as IBS [65]. In practice, there is no specific conventional medical therapy for either IBD or IBS and this has directed the treatment options towards relief of the predominant symptoms [4]. Most Chinese herbal formulas include a combination of several herbs with medicinal properties. Several studies on traditional herbal Chinese medicine have suggested the effectiveness of these therapies for treatment of IBS [66]. In a randomized, controlled trial assessing the effect of three Chinese herbal formulations on the patients suffering from IBS, a significant improvement was observed in patients taking the herbal therapy compared to the placebo group [67].

The natural products derived from medicinal plants are a rich source of biologically active compounds. There are approximately 500,000 plants species found around the world, of which only 1% have been investigated for the presence of novel bioactive compounds and just a small fraction of those investigated in randomized controlled trials [66].

Chen et al. suggested ginger as a likely effective treatment for diarrhoea caused by enterotoxigenic *E. coli* (ETEC) [68]. Further research into the active components of ginger identified zingerone

as the likely constituent responsible for the antidiarrheal activity by blocking the toxin receptor [68]. According to the FDA, ginger is recognised as a safe herb and in German monographs no known side effects or drug/herb interactions were reported [68]. Toxigenic strains of *C. difficile* produce toxins that are important in the pathogenesis of disease and therapies targeting toxin production or activity are ideal management strategies for the prevention of diarrhoea. Thus, natural products or commonly used herbs that have anti-toxin activity against other pathogens may show similar activity against *C. difficile* and this is worthy of investigation.

4. Discussion

While antibiotics such as metronidazole and vancomycin are the standard therapy for CDI, they may become obsolete overtime due to overuse leading to the emergence of resistant strains of *C. difficile* [19]. Another major drawback of antimicrobial treatment of CDI is the continued disruption of the gut microbiota and the increasing risk of disease recurrence [1,19]. It is the disruption of the normal gut flora that leads to *C. difficile* spores germinating, producing toxins and causing colitis [18]. Some studies suggest that the initial therapy for patients with a first episode of CDI who are at risk of recurrent disease should be fidaxomicin [69]. Fidaxomicin has a narrow spectrum activity and spares the majority of intestinal flora, however, its high cost compared to other therapeutic regimens may restrict its role.

One of the rapidly developing therapeutic options is FMT, especially in patient with recurrent CDI. However, more studies on safety and long-term side effects of FMT are required to reduce the possible transmission of infection and predisposition of immune diseases from donors [23,70]. To help mitigate this problem relatively, stool donation from established blood donors who have been previously screened may be preferable over close relatives who may fail to disclose underlying diseases [23].

In general, developing an effective vaccine against *C. difficile* and vaccinating the high risk populations such as elderly, hospitalised patients, and those requiring the prolonged use of antimicrobials, will greatly help prevent CDI [19]. However, one of the challenges relative to vaccine development is inducing a prolonged, fast, effective and protective immunity.

As mentioned earlier, several possible CAMs have been investigated or are still undergoing investigation. Yet, there are several potential therapeutic options that need further study. An ideal treatment of CDI needs to achieve three main goals; reduction of *C. difficile* burden and toxin production, restoration of normal microflora and assisting the host immune system [38]. When developing a new or alternative treatment strategy, not only the resolution of initial infection, but also reducing the risk of disease recurrence needs to be considered.

CDI may share a similar clinical presentation with various other gastrointestinal disorders [71]. IBD and IBS are two gastrointestinal disorders that can produce diarrhoeal symptoms similar to those experienced in CDI [72,73]. Pathogens such as Shiga toxin-producing *Escherichia coli* (STEC) and spore forming *Bacillus cereus* are two other common causes of infectious diarrhoea that share with *C. difficile* the ability to cause disease through the production of toxins in the infected host [74,75]. Investigating approaches that are used in treating other toxin-mediated diseases and share similarities with CDI may be worthwhile.

The rising emergence of antimicrobial-resistant strains encourages studying natural products such as herbal extracts and essential oils for treatment of CDI. Many natural products inhibit pathogens using mechanisms different from those employed by antimicrobials, making it difficult for pathogens to develop resistance [76].

5. Conclusions

C. difficile is an important cause of disease in both human and animals. While standard pharmacological antimicrobial treatment is a suitable therapeutic option for most mild to moderate cases of CDI, approximately 25% of patients experience the return of the symptoms or recurrence of the disease. Hence, evaluating CAM including a range of popular and novel compounds that are primarily used for the treatment of other diarrhoeal diseases for therapeutic action against CDI is worthwhile. Furthermore, future research should be directed towards investigation of further potential alternative therapies for this infection. This may initiate the introduction of new therapeutic options for treating CDI. Also, use of CAM has the potential to reduce the rate of growing resistant to antimicrobials, as well as the costs associated with this problem. However, more research is required, including on mechanisms of action, toxicological profiles, drug-drug interaction and effects on commensal flora to determine clinical usefulness of natural products.

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ORIGINAL ARTICLE

Effects of natural products on several stages of the spore cycle of *Clostridium difficile* in vitro

N. Roshan¹ , T.V. Riley^{1,2,3,4} and K.A. Hammer¹ ¹ School of Biomedical Sciences (M504), The University of Western Australia, Crawley, WA, Australia² Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia³ School of Veterinary & Life Sciences, Murdoch University, Murdoch, WA, Australia⁴ School of Medical & Health Sciences, Edith Cowan University, Joondalup, WA, Australia**Keywords**alternative therapy, *Clostridium difficile* spores, germination, outgrowth, sporulation.**Correspondence**

Katherine A. Hammer, School of Biomedical Sciences (M504), The University of Western Australia, 35 Stirling Hwy, Crawley, WA, Australia.

E-mail: katherine.hammer@uwa.edu.au

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Abstract**Aims:** To investigate the effect of natural products on the spore cycle of *Clostridium difficile* in vitro.**Methods and Results:** Twenty-two natural products were investigated using four *C. difficile* strains. Effects on sporulation, determined using microscopy and a conventional spore recovery assay, showed that fresh onion bulb extract (6.3% v v⁻¹) and coconut oil (8% v v⁻¹) inhibited sporulation in all four isolates by 66–86% and 51–88%, respectively, compared to untreated controls. Fresh ginger rhizome extract (25% v v⁻¹) was also inhibitory, although to a lesser extent. Using a standard spore germination and outgrowth assay, germination was unaffected by the 22 products, whereas outgrowth was significantly reduced by artichoke extract (18.8 mg ml⁻¹), fresh onion bulb extract (25% v v⁻¹), *Leptospermum* honeys (8% w v⁻¹) and allicin (75 mg ml⁻¹; *P* < 0.01). Sporocidal activity, investigated using a standard plate recovery assay, was minimal.**Conclusions:** Three of the 22 natural products (13%) showed inhibitory effects on sporulation of *C. difficile* and six products (27%) reduced vegetative outgrowth of *C. difficile*.**Significance and Impact of the Study:** This study shows the potential of natural products to inhibit different stages of *C. difficile* sporulation and encourages further investigation in this field.**Introduction**

Clostridium difficile, an anaerobic, Gram-positive, spore-forming bacillus, is the main cause of nosocomial and antibiotic-associated diarrhoea (Cheng *et al.* 2011; Surawicz and Alexander 2011). *Clostridium difficile* infection (CDI) has a variety of clinical manifestations ranging from asymptomatic carriage and mild/self-limiting diarrhoea to life-threatening sequelae, such as pseudomembranous colitis and toxic megacolon (Jump *et al.* 2007; Riley 2010). Antibiotic therapy and exposure to the organism are the main risk factors for acquiring CDI (Henrich *et al.* 2009). Toxigenic strains of *C. difficile* usually produce an enterotoxin (TcdA) and a cytotoxin (TcdB), that are the main virulence factors and cause

major damage to the intestinal epithelium (Riley 2010). Also, an additional toxin, binary toxin is produced by some strains of *C. difficile*; however, there is debate about the role of this toxin in disease (Cheng *et al.* 2011).

Clostridium difficile exists in both vegetative and spore forms. During infection, *C. difficile* begins a sporulation process, in which vegetative cells produce dormant endospores that can persist and disseminate in the environment, and become a source for disease transmission and recurrence (Paredes-Sabja *et al.* 2014). CDI can be treated primarily with the antibiotics vancomycin and metronidazole; however, approximately 25% of patients treated with either of these antibiotics will experience a recurrence of symptoms following treatment (Surawicz and Alexander 2011). Moreover, *C. difficile* spores are

metabolically dormant and show resistance to antibiotics, the host's immune system and bleach-free disinfectants (Baines *et al.* 2008; Paredes-Sabja and Sarker 2012). Thus, the *C. difficile* spore is the infective particle and antibacterial agents that have an effect against bacterial spores have become a focus for finding new anti-*C. difficile* therapies (Pyrek 2013).

Complementary and alternative therapies encompass a wide range of practices including but not limited to herbal remedies and nutritional supplements (McFarland 2005). Natural products derived from medicinal plants are a rich source of biologically active compounds; however, they are embraced less by pharmaceutical and medical professions (Palombo 2006). We have recently shown that several natural products have significant antimicrobial activity against *C. difficile* *in vitro* (Roshan *et al.* 2017). The aim of the present study was to determine the effect of those natural products tested previously on the spore cycle of *C. difficile*, on sporulation, spore germination and outgrowth of *C. difficile* spores. In addition, sporicidal activity was determined for those products showing bactericidal activity against vegetative cells.

Material and methods

Bacterial strains

The following four strains of *C. difficile* were examined in this study: *C. difficile* ATCC 700057 (A⁻B⁻CDT⁻), *C. difficile* NCTC 13366 (ribotype 027, A⁺B⁺CDT⁺), *C. difficile* R11446 (ribotype 014, A⁺B⁺CDT⁻, clinical isolate) and *C. difficile* ATCC 43598 (ribotype 017, A⁻B⁺CDT⁻). Strains were obtained from the School of Biomedical Sciences at The University of Western Australia and Path-West Laboratory Medicine, WA.

Antimicrobial agents

Twenty-two natural products were selected for testing based on historical evidence, their current popularity, and feasibility (Table 1). The products were categorized into three broad groups; raw products usually consumed as food, processed products taken as health supplements and the active pure compounds found in these products. The raw products ($n = 10$) were purchased from a supermarket in Perth, Western Australia (WA; Table 1) while the *Leptospermum* honeys were kindly provided by Capilano Company, Bayswater, WA. Processed and pure products ($n = 12$) were purchased from pharmacies and health food stores in Perth, WA, or from the manufacturers (Table 1). The products were prepared as previously described (Roshan *et al.* 2017).

Either fidaxomicin or vancomycin was used as a control in the sporulation and germination assays. Stock solutions of products in the form of powder or oil were prepared in 20% dimethyl sulfoxide (DMSO). Further dilutions were performed in sterile distilled water (SDW) for all products. Prior to testing, raw products were filtered through a 0.2 μm pore size filter to remove any microbial contamination, however, the processed products were not filtered as they were assumed to have had gone through a sterilization process during manufacturing.

Broth microdilution assay

Minimum inhibitory concentrations (MICs) for natural products and antimicrobial agents were determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines with slight modification (CLSI 2009).

MIC determination for log-phase *C. difficile* cultures used in sporulation study

Initially, growth from a 48 h culture of each *C. difficile* strain on blood agar (BA) was suspended in prereduced brain heart infusion broth (BHIB) and incubated at 35°C in an anaerobic chamber (Don Whitley Scientific Pty Ltd, Otley, West Yorkshire) until the cells reached midexponential phase (approximately 12 h, OD₆₀₀ $\approx$ 0.5; data not shown). Liquid medium containing 3% tryptose and 2% yeast extract (TY) was used for the broth microdilution assay to mimic the conditions used in the subsequent sporulation assay. Briefly, midlog phase cultures of *C. difficile* were inoculated into TY broth containing a range of concentrations of natural products and antimicrobial agents in a 96-well tray to give a final inoculum concentration of approximately 1×10^6 CFU per ml. The total volume for each test was 200 μl per well with a 1 : 1 proportion of cells and test compounds. Trays were incubated anaerobically at 35°C for 48 h and MICs were determined visually with the aid of a reading mirror at 24 and 48 h as the lowest concentration of product preventing growth and resulting in no turbidity. Also, doubling dilutions of 20% DMSO were prepared and tested in parallel (solvent control) and fidaxomicin was included as an antibiotic control.

MIC and minimum bactericidal concentration determination for stationary-phase *C. difficile*

Clostridium difficile strains were primarily cultured on prereduced Brucella agar supplemented with 5 μg hemin, 1 μg vitamin K₁ and 5% (v v⁻¹) laked sheep blood and

Natural products and CDI

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Table 1 Natural products investigated in the study

Product	Concentration	Ingredient information	Supplier
Raw products			
Fresh garlic bulb	1 ml ml ⁻¹	None	–
Fresh onion bulb	1 ml ml ⁻¹	None	–
Fresh ginger rhizome	1 ml ml ⁻¹	None	–
<i>Leptospermum</i> honey (NPA 15+) (A)	100% pure Manuka honey	None	Barnes Naturals, Richlands, Qld, Australia
<i>Leptospermum</i> honey (NPA 10+) (B)	100% pure Manuka honey	None	Barnes Naturals
<i>Leptospermum</i> honey (NPA 10+) (C)	100% pure Manuka honey	None	Barnes Naturals
Garlic clove powder	1 mg mg ⁻¹	None	Spencers, Fremantle, WA, Australia
Ginger rhizome powder	1 mg mg ⁻¹	None	Spencers
Cinnamon root powder	1 mg mg ⁻¹	None	Spencers
Turmeric root powder	1 mg mg ⁻¹	None	Spencers
Processed products			
Garlic tablet	Garlic (<i>Allium sativum</i>) extract equivalent to dry bulb 10 000 mg	No added yeast, starch, gluten, lactose, sugar, artificial colours or flavours, artificial sweeteners or preservatives, dairy products or animal-derived products	Nature's Own, Virginia, Qld, Australia
Ginger tablet	1000 mg	No added yeast, starch, gluten, lactose, sugar, artificial colours or flavours, artificial sweeteners or preservatives, dairy products or animal-derived products	Nature's Own
Cinnamon tablet	Cinnamon (<i>Cinnamomum cassia</i>) stem bark inner powder 1000 mg	No added lactose, egg, gluten, soy, yeast, artificial colours and artificial flavours	Swisse, Collingwood, Vic., Australia
Turmeric tablet	1000 mg	Turmeric powder 77%, cellulose microcrystalline, povidone, silica colloidal anhydrous, magnesium stearate, croscarmellose sodium	Nature's Way, Warriewood, NSW, Australia
Artichoke capsule	Herbal extract equivalent to <i>Cynara scolymus</i> fresh leaf 60 000 mg	No added lactose, starch, added sugars and salt, artificial colourings and flavourings or preservatives	Nature's Sunshine, Baulkham hills, NSW, Australia
Coconut oil capsule	100% coconut oil	Capsule shell (gelatin, glycerol, water)	Nature's Way
Peppermint oil	100% pure, <i>Mentha X piperita</i> leaf 1 ml ml ⁻¹	None	Oil Garden, Springvale, Vic., Australia
Aloe vera gel	99% pure <i>Aloe Barbadensis</i>	Allantoin (found in Comfrey root), carbomer, disodium edetate, sodium hydroxyl methylglycinate, grapefruit seed extract	Plunkett's, Warriewood, NSW, Australia
Pure compounds			
Allicin	180 mg allicin extract (garlic bulb)	Vegetarian capsule (hypromellose, water), maltodextrin, gum acacia, Made without corn, wheat, gluten, yeast, soy, sugar, dairy, artificial colours or flavours	Vcaps, Chicago, IL, USA

(Continued)

Table 1 (Continued)

Product	Concentration	Ingredient information	Supplier
<i>trans</i> -Cinnamaldehyde	Purity >98.5%	None	Sigma Aldrich, Saint Louis, MO, USA
Menthol	Purity >98.5%	None	Sigma Aldrich
Zingerone	Purity >96%	None	Sigma Aldrich

NPA, nonperoxide activity.

were incubated anaerobically for 48 h at 35°C. A suspension containing 1×10^6 CFU per ml was prepared for each strain in prereduced supplemented Brucella broth and was used to inoculate serial dilutions of natural products and antimicrobial agents prepared in 96-well microtitre trays. The total volume for each well was 200 μ l with a 1 : 1 proportion of cells and test compounds. After incubation for 48 h, the MIC values were determined as above. The minimum bactericidal concentration (MBC) was determined by spot-inoculating 10 μ l volumes from nonturbid wells onto prereduced BA. Agar plates were incubated for 24–48 h anaerobically and MBCs determined as the lowest concentration preventing growth. As above, serial twofold dilutions of 20% DMSO were prepared and tested against all strains in each experiment.

Sporulation in liquid broth cultures

This assay was performed as described previously (Garneau *et al.* 2014) with slight modifications. Log-phase cultures of *C. difficile* were inoculated into TY broth containing 0.5 $\times$ MIC treatments, to result in a final concentration of 1×10^6 CFU per ml (Table 2). The assay was carried out in 96-well microtitre trays with a total volume of 200 μ l per well. Untreated *C. difficile* controls were also included. Duplicate microtitre trays were incubated anaerobically at 35°C for 96 h. After 48 and 96 h of incubation, 50 μ l aliquots from each well were serially diluted and plated on BHI agar (BHIA) supplemented with 0.1% taurocholate and 1 mmol l⁻¹ glycine (BHI-tag) to count total CFUs. Also, 50 μ l aliquots from each sample were added to 50 μ l of 100% ethanol, treated for 1 h to kill any vegetative cells (50% final concentration) and then plated on BHI-tag to quantify viable spores. The mean CFU per ml from three experiments was calculated and the percentage of spores was determined by dividing the number of spores by the total count. To monitor the viability of vegetative cells, another 50 μ l volume from each sample was subcultured on BHIA without taurocholate and glycine, as spores germinate poorly in the absence of germinants.

Microscopic evaluation of spore formation

This assay was performed as described previously (Garneau *et al.* 2014). Briefly, aliquots of 15 μ l of each bacterial suspension grown in TY broth from the sporulation assay above were deposited onto microscope slides, air-dried and heat fixed at 80°C. Cells were stained with 0.5% aqueous safranin and malachite green and observed with a light microscope. For each slide, vegetative cells and spores were counted for five different fields of view, each containing at least 30 cells (minimum of 150 cells per slide). The percentage of spores was calculated for each experiment and the mean and standard deviation (SD) for three independent replicates were determined. All natural products were initially tested against *C. difficile* NCTC 13366 and spores and CFUs counted using both the conventional spore recovery assay and microscopic methods. Products that showed activity against *C. difficile* NCTC 13366 were tested further against the remaining three *C. difficile* strains. Fidaxomicin was used as a positive control (inhibiting spore formation), and SDW and the highest concentration of DMSO used in the experiment were included as untreated, negative controls (no effect on spore formation).

Spore germination and outgrowth assay

Spore suspensions were prepared according to the method described by Moore *et al.* (2013) with slight modification. Spore suspensions from all four strains were prepared by culturing *C. difficile* strains for 48 h on BA then subculturing onto prereduced chocolate agar and incubating anaerobically at 35°C for 10 days. Then, cultures were left at room temperature for 24 h to kill any vegetative cells. Growth from each plate was then suspended in cold SDW. To ensure that all vegetative cells were dead, the bacterial suspensions were incubated at 70°C in a water bath for 20 min. Subsequently, the suspensions were centrifuged at 4000 g for 10 min, and the pellet washed at least 10 times in SDW and resuspended in SDW. The purity of the spore suspension was determined using phase-contrast microscopy to ensure a spore purity of >95% and a spore count was performed by

Table 2 MICs of natural products against stationary and mid-exponential phase cultures of *Clostridium difficile*

Antimicrobial agents	MIC or MBC	Primary solvent	C. difficile NCTC 13366				C. difficile R11446				C. difficile ATCC 43598				C. difficile ATCC 70057				Concentration used for germination and outgrowth assay ¹
			Exponential		Stationary		Exponential		Stationary		Exponential		Stationary		Exponential		Stationary		
Raw products																			
Fresh garlic bulb extract % (v v ⁻¹)	MIC	–	0.4	0.8	0.8	0.4	0.4	0.8	0.8	0.4	0.4	0.8	0.4	0.8	0.8	0.8	50	50	50
Fresh ginger rhizome extract % (v v ⁻¹) ^a	MBC	–	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	>50	50
Fresh onion bulb extract % (v v ⁻¹) ^a	MIC	–	25	>50	>50	25	25	>50	>50	25	25	>50	25	>50	>50	>50	50, 25, 12.5 ²	>50	50, 25, 12.5 ²
Leptospermum honey (A) % (w v ⁻¹) ^{b, **}	MBC	SDW	4	16	16	4	4	16	16	4	4	16	4	16	16	4	16	32, 16, 8 ²	32, 16, 8 ²
Leptospermum honey (B) % (w v ⁻¹) ^{b, **}	MIC	SDW	4	16	16	4	4	16	16	4	4	16	4	16	16	4	16	32, 16, 8 ²	32, 16, 8 ²
Leptospermum honey (C) % (w v ⁻¹) ^{b, **}	MBC	SDW	8	32	32	8	8	32	32	8	8	32	8	32	32	8	32	32, 16, 8 ²	32, 16, 8 ²
Garlic clove powder (mg ml ⁻¹) ^c	MIC	20% DMSO	2.3	9.4	9.4	2.3	2.3	9.4	9.4	2.3	2.3	9.4	2.3	9.4	9.4	2.3	9.4	75	75
Ginger rhizome powder (mg ml ⁻¹) ^c	MIC	20% DMSO	75	>150	>150	75	37.5	>150	>150	37.5	37.5	>150	37.5	>150	>150	>150	>150	75	75
Cinnamon root powder (mg ml ⁻¹) ^c	MIC	20% DMSO	18.8	75	75	9.4	9.4	75	75	9.4	9.4	75	9.4	75	75	9.4	75	75	75
Turmeric root powder (mg ml ⁻¹) ^c	MBC	20% DMSO	75	>150	>150	37.5	37.5	>150	>150	37.5	37.5	>150	37.5	>150	>150	37.5	>150	75	75
Processed products																			
Garlic tablet (mg ml ⁻¹) ^c	MIC	20% DMSO	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	75	75
Ginger tablet (mg ml ⁻¹) ^c	MBC	20% DMSO	75	>150	>150	75	75	>150	>150	75	75	>150	75	>150	>150	75	>150	75	75
Cinnamon tablet (mg ml ⁻¹) ^c	MIC	20% DMSO	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	18.8	75	75
Turmeric tablet (mg ml ⁻¹) ^c	MBC	20% DMSO	75	>150	>150	37.5	37.5	>150	>150	75	75	>150	75	>150	>150	75	>150	75	75
Processed products																			
Artichoke capsule (mg ml ⁻¹) ^c	MIC	20% DMSO	18.8	75	75	9.4	9.4	75	75	9.4	9.4	75	9.4	75	75	9.4	75	75, 37.5, 18.8 ²	75, 37.5, 18.8 ²
Aloe vera gel % (w v ⁻¹) ^b	MBC	20% DMSO	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	32	32

(Continued)

Table 2 (Continued)

Antimicrobial agents	MIC or MBC	Primary solvent	C. difficile NCTC 13366		C. difficile R11446		C. difficile ATCC 43598		C. difficile ATCC 700057		Concentration used for germination and outgrowth assay ¹
			Exponential	Stationary	Exponential	Stationary	Exponential	Stationary	Exponential	Stationary	
Coconut oil % (v v ⁻¹) ^b	MIC	20% DMSO	32	>32	32	>32	32	>32	32	>32	32
	MBC			>32		>32		>32		>32	
Peppermint oil % (v v ⁻¹) ^b	MIC	20% DMSO	1	8	1	8	1	8	1	8	32
	MBC			8		8		8		8	
<i>Pure compounds</i>											
Allicin (mg ml ⁻¹) ^c	MIC	20% DMSO	4.7	4.7	2.3	2.3	4.7	4.7	4.7	4.7	75, 37.5, 18.8 ²
	MBC			>75		>75		>75		>75	
trans-Cinnamaldehyde % (v v ⁻¹) ^b	MIC	20% DMSO	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	32
	MBC			0.02		0.02		0.02		0.02	
Menthol (mg ml ⁻¹) ^c	MIC	20% DMSO	2.3	9.4	2.3	9.4	2.3	9.4	2.3	9.4	75
	MBC			18.8		18.8		18.8		18.8	
Zingerone (mg ml ⁻¹) ^c	MIC	20% DMSO	4.7	9.4	4.7	9.4	4.7	9.4	4.7	9.4	75
	MBC			9.4		18.8		9.4		9.4	
<i>Control</i>											
Fidaxomicin (µg ml ⁻¹) ^d	MIC	SDW	0.125	0.25	0.125	0.125	0.125	0.25	0.125	0.125	16, 8, 4 ²
	MBC			0.25		0.125		0.25		0.125	
Vancomycin (µg ml ⁻¹) ^d	MIC	SDW	ND	1	ND	0.5	ND	0.5	ND	0.5	16
	MBC			>16		>16		>16		>16	

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; SDW, sterile distilled water; NPA, nonperoxide activity; ND, not done.

*Serial twofold dilutions were performed in SDW for all the products.

***Leptospermum* honey (A), NPA 15⁺; *Leptospermum* honey (B), NPA 10⁺; *Leptospermum* honey (C), NPA 10⁺.Dilutions starting at ^a50, ^b32, ^c150, ^d16.¹In some instances, concentrations could not be supra-MIC due to DMSO or not showing a MIC value in the range of concentration of treatments used.²For the products that showed an effect, the assay was repeated with twofold and fourfold lower concentrations. Dash means no solvent.

serially diluting and plating the spore suspensions on Chrom-ID *C. difficile* agar (bioMérieux, Marcy-l'Étoile, France). Spore suspensions were then stored at -20°C until use.

To examine effects on spore germination, spores of all four *C. difficile* strains were each suspended to a final concentration of 1×10^6 CFU per ml in prereduced BHIB supplemented with 5 g l^{-1} yeast extract, 0.1% L-cysteine and 0.1% taurocholate containing natural products at supra-MIC concentrations (Table 3). The assay was performed as previously described (Allen *et al.* 2013). Spore germination and outgrowth was measured by determining the absorbance at 600 nm (A_{600}) at time zero, every 5 min for the first 30 min, every 10 min for the next 30 min and then every 1 h for another 6 h and finally at 24 h using a Bio-Rad xMark spectrophotometer (Hercules, CA, USA). The assay was performed in 96-well trays that were incubated anaerobically at 35°C between measurements. The assay was repeated at twofold and fourfold lower concentrations for treatments that showed an effect on either the germination or the outgrowth stage. Germination and outgrowth were normalized to time zero (t_0) by dividing the optical density (OD) measured at each time point by the OD at time zero.

Fidaxomicin and vancomycin were included as positive controls (inhibiting *C. difficile* outgrowth), and both SDW and the highest concentration of DMSO used in the experiment were included as untreated controls (affecting neither *C. difficile* germination nor outgrowth).

Sporicidal activity

Sporicidal activity was evaluated as previously described (Lawrence and Palombo 2009). Spore suspensions prepared as described above were diluted in 0.85% saline to result in a final concentration of 1×10^6 CFU per ml after the addition of treatments. The final volume for each spore suspension was 5 ml after the addition of treatments. Suspensions were incubated anaerobically at 35°C for 24 h, 48 h and 1 week. After incubation, 1 ml aliquots were removed and spores were recovered by centrifuging suspensions for 10 min at 4000 g and washing twice in 0.85% saline to minimize carryover of any residual products. The washed spore pellets were suspended in 1 ml of 0.85% saline, serially diluted and plated on BHI-tag agar. The number of CFUs was determined after 24–48 h incubation anaerobically at 35°C . Negative controls consisted of SDW and the highest concentration of DMSO. The limit of detection was 100 CFU per ml for this assay.

Statistical analysis

All assays were repeated three times. A modal value was determined for MIC and MBCs obtained by broth

microdilution assay. The mean and SD was calculated for the number of CFUs in sporulation and sporicidal assays and for the number of spores and vegetative cells observed in the microscopic evaluation of spore formation. The mean and SD was calculated for normalized OD ratios in the germination assay. Statistical differences between three or more sets of data were analysed in GraphPad Prism v.7 (La Jolla, CA, USA) software using one-way analysis of variance and nonparametric technique, followed by Tukey's multiple comparison post-test if the *P* value was significant. Student's two-tailed *t*-test, assuming unequal variance was used to determine whether there was a significant difference between two sets of data. *P* values of < 0.05 were considered significant.

Results

Antimicrobial susceptibility testing

The susceptibility of exponential and stationary-phase cultures of all four strains of *C. difficile* to antimicrobial agents is shown in Table 2. All natural products except fresh ginger rhizome extract showed activity against all four midexponential phase *C. difficile* strains with *trans*-cinnamaldehyde being the most active product. Stationary-phase *C. difficile* strains were less susceptible to the products compared to midexponential phase culture and had higher MIC values, but *trans*-cinnamaldehyde was still the most active product. Based on MBC values determined for stationary-phase *C. difficile*, only five products showed bactericidal activity (Table 2). MICs for antimicrobial agents were within the expected ranges (Table 2). For the DMSO solvent control, the 10% and 5% DMSO showed inhibitory activity against midexponential phase *C. difficile* strains but no effect was observed with 2.5% DMSO or lower. For stationary-phase *C. difficile* cultures, 10% DMSO showed some inhibitory and bactericidal activity, but no effect was observed with 5% DMSO or lower.

Sporulation assays

The effect of $0.5 \times \text{MIC}$ of each natural product on the production of spores by *C. difficile* NCTC 13366 is shown in Table 3. The growth rate of bacteria treated with $0.5 \times \text{MIC}$ was compared to untreated controls by measuring OD_{600} to ensure that $0.5 \times \text{MIC}$ did not significantly inhibit growth and that similar growth was achieved (Bio-Rad xMark spectrophotometer). Growth was not significantly retarded by $0.5 \times \text{MIC}$. The proportion of spores for each treatment was compared with the proportion of spores for the untreated and solvent

controls (SDW and DMSO). For *C. difficile* NCTC 13366, fresh ginger rhizome extract (50% v v⁻¹), fresh onion bulb extract (12.5% v v⁻¹) and coconut oil (16% v v⁻¹) at 0.5 × MIC were the only treatments that significantly reduced spore production in both assays after 48 and 96 (P < 0.001; Table 3; Fig. 1). Numbers of spores from the remaining treatments did not differ significantly from the untreated controls. Fresh ginger rhizome extract, fresh onion bulb extract and coconut oil were also tested at 0.25 × MIC against *C. difficile* NCTC 13366. These three products in addition to the controls, were assessed against the three remaining *C. difficile* strains ATCC 700057, ATCC 43598 and R11446 at both 0.5 × MIC and 0.25 × MIC (Fig. 1 and Fig. S1). These three treatments at 0.5 × MIC significantly inhibited spore formation in all four *C. difficile* strains by 50–90% (P ≤ 0.042) with the exception of fresh ginger rhizome extract, which did not significantly reduce spore formation for *C. difficile* ATCC 43598 after either 48 or 96 h and had no

inhibitory effect at 96 h for *C. difficile* ATCC 700057. Fresh onion bulb extract at 0.25 × MIC (6.3% v v⁻¹) significantly inhibited spore formation in all four *C. difficile* strains compared to the untreated and solvent controls (P ≤ 0.041). Coconut oil at 0.25 × MIC (8% v v⁻¹) also inhibited spore formation in most strains except for *C. difficile* strains ATCC 43598 at 96 h growth (P ≤ 0.045). Fresh ginger rhizome extract at a concentration of 25% v v⁻¹ only inhibited spore formation for *C. difficile* strains NCTC 13366, ATCC 700057 and R11446 after 48 h growth (P ≤ 0.019).

Spore germination and outgrowth assay

None of the products affected spore germination by any *C. difficile* isolate at concentrations that were mostly suprainhibitory for stationary-phase *C. difficile* (Fig. 2). Suspensions of spores in supplemented BHIB showed a decrease in A₆₀₀ until approximately 15 min to 2 h

Table 3 Proportion of spores of *Clostridium difficile* NCTC 13366 after 48 and 96 h of growth in TY broth containing 0.5 × MIC of natural products

Antimicrobial agents (concentration)	% spores ± SD			
	Conventional spore recovery assay		Microscopy	
	48 h	96 h	48 h	96 h
Fresh garlic bulb extract (0.2% v v ⁻¹)	8.6 ± 0.5	17.9 ± 1.2	17.0 ± 2.8	29.4 ± 2.9
Fresh ginger rhizome (50% v v ⁻¹)	1.6 ± 0.4*	3.2 ± 0.3*	7.0 ± 2.1*	10.5 ± 2.1*
Fresh onion bulb extract (12.5% v v ⁻¹)	0.8 ± 0.1*	4.2 ± 0.3*	4.2 ± 0.4*	7.6 ± 1.1*
<i>Leptospermum</i> honey (A) (4% w v ⁻¹)	7.6 ± 0.5	18.3 ± 2.0	16.9 ± 1.9	30.3 ± 2.7
<i>Leptospermum</i> honey (B) (4% w v ⁻¹)	8.1 ± 2.4	17.6 ± 1.3	18.4 ± 3.8	32.0 ± 2.5
<i>Leptospermum</i> honey (C) (4% w v ⁻¹)	9.5 ± 1.4	18.1 ± 1.4	17.2 ± 2.8	31.6 ± 2.1
Garlic clove powder (1.2 mg ml ⁻¹)	7.9 ± 0.7	16.8 ± 1.6	19.1 ± 2.5	31.0 ± 3.0
Ginger rhizome powder (37.5 mg ml ⁻¹)	7.8 ± 1.3	16.2 ± 1.7	18.3 ± 2.7	28.8 ± 3.1
Cinnamon root powder (9.4 mg ml ⁻¹)	9.6 ± 0.6	18.5 ± 2.8	20.1 ± 2.3	32.8 ± 2.7
Turmeric root powder (37.5 mg ml ⁻¹)	7.1 ± 0.9	18.1 ± 1.3	16.3 ± 2.3	27.1 ± 3.8
Garlic tablet (18.8 mg ml ⁻¹)	7.6 ± 0.1	15.9 ± 0.7	20.3 ± 2.1	30.4 ± 2.8
Ginger tablet (37.5 mg ml ⁻¹)	8.4 ± 0.6	14.0 ± 2.5	19.5 ± 1.5	28.9 ± 3.4
Cinnamon tablet (9.4 mg ml ⁻¹)	9.7 ± 0.6	15.1 ± 1.5	18.4 ± 3.1	36.0 ± 2.5
Turmeric tablet (37.5 mg ml ⁻¹)	7.1 ± 0.9	15.1 ± 1.3	18.5 ± 1.9	34.3 ± 3.4
Artichoke capsule (9.4 mg ml ⁻¹)	7.4 ± 1.6	16.4 ± 1.2	16.7 ± 1.1	32.0 ± 3.6
Aloe vera (8% w v ⁻¹)	9.0 ± 0.4	13.5 ± 2.7	17.6 ± 3.2	34.1 ± 3.8
Coconut oil (16% v v ⁻¹)	2.8 ± 0.7*	4.2 ± 0.7*	4.1 ± 1.3*	6.4 ± 1.0*
Peppermint oil (1% v v ⁻¹)	9.6 ± 0.7	17.3 ± 2.4	16.1 ± 1.9	30.3 ± 3.9
Allicin (2.3 mg ml ⁻¹)	8.0 ± 0.9	15.5 ± 2.4	15.9 ± 2.6	27.1 ± 3.5
<i>trans</i> -Cinnamaldehyde (0.001% v v ⁻¹)	8.3 ± 0.4	15.7 ± 1.3	17.4 ± 3.3	30.5 ± 2.6
Menthol (2.3 mg ml ⁻¹)	7.7 ± 0.2	13.5 ± 2.8	15.7 ± 3.5	32.7 ± 3.4
Zingerone (4.7 mg ml ⁻¹)	7.8 ± 0.3	16.5 ± 3.2	15.7 ± 3.6	28.4 ± 2.8
Fidaxomicin (0.125 mg ml ⁻¹)	0.6 ± 0.3*	2.5 ± 0.4*	1.2 ± 0.8*	3.0 ± 0.7*
Untreated control (SDW)	8.8 ± 0.9	17.4 ± 1.5	19.4 ± 2.7	33.8 ± 1.9
Untreated control (DMSO) (2.5% v v ⁻¹)	8.0 ± 0.9	19.2 ± 1.9	16.2 ± 2.9	32.3 ± 3.6

SD, standard deviation; h, hour; SDW, sterile distilled water; DMSO, dimethyl sulfoxide. *Leptospermum* honey (A), NPA 15+; *Leptospermum* honey (B), NPA 10+; *Leptospermum* honey (C), NPA 10+.

Differ significantly from the untreated controls (SDW and DMSO): *P < 0.001.

Figure 1 The percentage of spores after 48 and 96 h growth in liquid TY broth containing $0.5 \times \text{MIC}$ of natural products or antibiotics determined by conventional spore recovery and microscopy. Concentrations of agents used in the assay: Onion juice ($12.5\% \text{ v v}^{-1}$); Ginger juice ($50\% \text{ v v}^{-1}$); Coconut oil ($16\% \text{ v v}^{-1}$); Fidaxomicin ($0.06 \mu\text{g ml}^{-1}$) for *Clostridium difficile* ATCC 700057, ATCC 43598 and R44116; Fidaxomicin ($0.125 \mu\text{g ml}^{-1}$) for *C. difficile* NCTC 13366; DMSO ($2.5\% \text{ v v}^{-1}$). ■ = $P < 0.05$, ♦ = $P < 0.01$, * = $P < 0.001$. (■) Conventional spore recovery assay; (■) Microscopy.

(depending on the strain), which indicated germination of spores. At approximately 20 min, there was an increase in absorbance for the majority of spore suspensions containing treatments, which correlated with the conversion of dormant spores to germinated spores and the start of vegetative outgrowth. Spore suspensions of all four isolates treated with artichoke extract (75 mg ml^{-1}), fresh onion bulb extract ($50\% \text{ v v}^{-1}$), three *Leptospermum* honeys ($32\% \text{ v v}^{-1}$) and allicin (75 mg ml^{-1}) showed no significant increase in absorbance from 7 to 24 h ($P \geq 0.691$) and absorbance at 24 h differed significantly from the untreated controls ($P \leq 0.05$). The assay was repeated using twofold and fourfold lower concentrations of these six products, and fidaxomicin. Similar results were obtained at lower concentrations for both artichoke extract and the three *Leptospermum* honeys (Figs S2 and S3). The exception was fresh onion bulb extract at the fourfold lower concentration of 12.5% , which showed a significantly higher absorbance (indicating outgrowth) at 24 h compared to 7 h ($P \leq 0.025$) and allicin at both twofold and fourfold lower concentrations, which showed a significant outgrowth at 24 h compared to 7 h ($P \leq 0.029$). Therefore, it was concluded that the late stage of *C. difficile* outgrowth remained constant, and suppressed, in the presence of these treatments at certain concentrations. The remaining treatments, including controls (SDW and DMSO), showed a significant increase of outgrowth from 7 h to 24 h for all four *C. difficile*

isolates ($P \leq 0.05$; data not shown). As previously reported, fidaxomicin and vancomycin inhibit the outgrowth of *C. difficile* spores at concentrations that are suprainhibitory for vegetative growth (Allen et al. 2013). In this study, these two antimicrobials did not inhibit the initiation of outgrowth, but outgrowth continued at reduced rates and for some strains remained low until approximately 5 h, followed by a nonsignificant decrease in outgrowth from 7 to 24 h ($P \geq 0.078$). The OD at 24 h for the antibiotic controls was significantly lower when compared to the untreated controls ($P \leq 0.001$).

Sporicidal activity of natural products

Of the five products tested for sporicidal activity against *C. difficile*, peppermint oil and *trans*-cinnamaldehyde exhibited some level of activity compared to the untreated controls (Tables 4 and 5). Based on the initial testing at the highest possible concentration of products, peppermint oil ($32\% \text{ v v}^{-1}$) and *trans*-cinnamaldehyde ($32\% \text{ v v}^{-1}$) showed significant sporicidal activity at all three exposure times (24 h, 48 h and 1 week) compared to the untreated controls ($P < 0.01$). Peppermint oil and *trans*-cinnamaldehyde were further tested at lower concentrations against two strains, *C. difficile* NCTC 13366 and ATCC 700057 (Table 5). In general, a long exposure time and a high concentration of product was required to show substantial sporicidal activity.

Figure 2 Effect of natural products on germination and outgrowth of *Clostridium difficile* spores. Concentration of agents used in the assay: DMSO (2.5% v v⁻¹), Fidaxomicin (16 µg ml⁻¹), Artichoke (75 mg ml⁻¹), Fresh onion bulb extract (50% v v⁻¹), *Leptospermum* honeys A, B and C (32% w v⁻¹), Allicin (75 mg ml⁻¹). (—●—) SDW; (—■—) DMSO; (—▲—) Fidaxomicin; (—▼—) Artichoke; (—◆—) Fresh onion extract; (—○—) *Leptospermum* honey (A); (—□—) *Leptospermum* honey (B); (—△—) *Leptospermum* honey (C); (—▽—) Allicin.

Discussion

The aim of this study was to investigate the *in vitro* impact of various natural products on different stages of the spore cycle of *C. difficile*. The sporulation assay suggested that fresh onion bulb extract and coconut oil, and to a lesser extent fresh ginger rhizome extract, inhibited the sporulation of *C. difficile*; however, the effect varied across strains. Trends observed by the conventional spore recovery assay and by microscopy were similar. Each sporulation experiment included a control assessing the viability of vegetative cells, and therefore the possibility that the inhibition of sporulation might have been due to a growth defect or death of vegetative cells could be eliminated. Higher numbers of spores were observed using microscopy compared to the conventional spore recovery method, which could be due to differences in methodology or nonviability of some spores; however, similar trends were observed by both techniques.

Several previous studies have investigated the inhibitory effects of antimicrobials on spore formation by *C. difficile* (Babakhani et al. 2012; Garneau et al. 2014). Garneau et al. (2014) reported the inhibitory effect of tigecycline and piperacillin/tazobactam on spore formation by several *C. difficile* isolates (Garneau et al. 2014). Fidaxomicin at a subinhibitory concentration also inhibited spore production by approximately 50% for *C. difficile* strain ATCC 4355 and a ribotype 014 isolate (Babakhani et al. 2012). In the same study, suprainhibitory concentrations of fidaxomicin resulted in a lower transcription of sporulation-specific mRNAs. In our study, fidaxomicin was used as a positive control and its inhibitory effect on spore formation was similar to that reported earlier (Babakhani et al. 2012). In a previous study, carvacrol at sub-MIC significantly reduced spore formation of *C. difficile* and down-regulated the genes involved in spore production ($P < 0.05$). Also, the MIC of carvacrol completely inhibited the spore outgrowth (Mooyottu et al. 2017). However, to the best of our knowledge, only

Table 4 Sporicidal activity of selected natural products against *Clostridium difficile* isolates

Antimicrobial agent (concentration)	Incubation time	Log reduction in viable spores			
		<i>C. difficile</i> NCTC 13366	<i>C. difficile</i> R11446	<i>C. difficile</i> ATCC 43598	<i>C. difficile</i> ATCC 700057
Cinnamon root powder (75 mg ml ⁻¹)	24 h	0.14 ± 0.08	0.08 ± 0.10	0.12 ± 0.06	0.06 ± 0.04
	48 h	0.21 ± 0.08	0.13 ± 0.08	0.23 ± 0.04	0.16 ± 0.07
	7 d	0.13 ± 0.09	0.17 ± 0.09	0.32 ± 0.14	0.12 ± 0.10
Peppermint oil (32% v v ⁻¹)	24 h	1.22 ± 0.42***	0.99 ± 0.11***	1.19 ± 0.20***	1.2 ± 0.23***
	48 h	1.72 ± 0.12***	1.20 ± 0.21***	0.98 ± 0.28**	2.00 ± 0.28***
	7 d	1.98 ± 0.37***	1.86 ± 0.40***	1.72 ± 0.31***	1.78 ± 0.48***
<i>trans</i> -Cinnamaldehyde (32% v v ⁻¹)	24 h	1.09 ± 0.34***	1.10 ± 0.26***	0.92 ± 0.49**	1.22 ± 0.08***
	48 h	1.75 ± 0.23***	1.13 ± 0.41***	1.36 ± 0.37***	1.87 ± 0.34***
	7 d	2.25 ± 0.12***	2.22 ± 0.26***	1.99 ± 0.27***	2.17 ± 0.43***
Menthol (75 mg ml ⁻¹)	24 h	0.18 ± 0.16	0.23 ± 0.10	0.22 ± 0.13	0.20 ± 0.07
	48 h	0.46 ± 0.16	0.50 ± 0.17	0.40 ± 0.20	0.49 ± 0.27
	7 d	0.48 ± 0.09	0.36 ± 0.14	0.48 ± 0.08	0.57 ± 0.05
Zingerone (75 mg ml ⁻¹)	24 h	0.20 ± 0.17	0.18 ± 0.03	0.24 ± 0.09	0.33 ± 0.11
	48 h	0.27 ± 0.19	0.23 ± 0.18	0.37 ± 0.15	0.19 ± 0.08
	7 d	0.53 ± 0.26	0.48 ± 0.11	0.46 ± 0.07	0.58 ± 0.25
SDW	24 h	0.08 ± 0.11	0.05 ± 0.06	0.09 ± 0.02	0.10 ± 0.09
	48 h	0.12 ± 0.07	0.07 ± 0.02	0.08 ± 0.10	0.09 ± 0.01
	7 d	0.12 ± 0.08	0.10 ± 0.05	0.07 ± 0.04	0.08 ± 0.02
DMSO (2.5% v v ⁻¹)	24 h	0.07 ± 0.07	0.07 ± 0.05	0.03 ± 0.12	0.09 ± 0.03
	48 h	0.08 ± 0.05	0.05 ± 0.02	0.06 ± 0.07	0.06 ± 0.03
	7 d	0.15 ± 0.08	0.13 ± 0.08	0.13 ± 0.06	0.06 ± 0.01

SDW, sterile distilled water; DMSO, dimethyl sulfoxide; h, hour; d, day.

Statistical significance: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to the untreated controls (SDW and DMSO).

a few studies have reported the effects of natural products on spore formation of *C. difficile* and other spore-forming pathogens (Chalfoun *et al.* 2004; Pandey *et al.* 2015).

When bacterial spores contaminate the environment, they remain viable for months to years and are difficult to eradicate as they are resistant to common disinfectants (Paredes-Sabja *et al.* 2014). Spores can spread through patient to patient contact and by environmental contamination, and this may result in new and recurrent infections (Gerding *et al.* 2008; Paredes-Sabja *et al.* 2014). Therefore, treatments preventing spore formation that are given at the same time as conventional antibiotics could reduce the possibility of transmission and the risk of recurrences. Also, some of these products may be suitable for use in the environment to prevent the formation of spores and thus reduce the risk of infection.

Germination of *C. difficile* spores is an important factor in the initiation of CDI as it is the first step after colonization (Moore *et al.* 2013; Paredes-Sabja *et al.* 2014). The germination process is required in order to allow outgrowth of vegetative cells (Moore *et al.* 2013). The current study showed that none of the products inhibited spore germination; however, treatment with artichoke extract, fresh onion bulb extract, *Leptospermum* honey and allicin at certain concentrations kept

the outgrowth constant from 7 to 24 h, which suggests inhibition of the late stage of vegetative growth compared to controls. Two previous studies assessing the effect of antimicrobials including fidaxomicin, vancomycin, oritavancin and metronidazole on *C. difficile* spore germination and outgrowth did not observe any effect on initiation of spore germination in the presence of suprainhibitory concentrations of any of the antimicrobials (Allen *et al.* 2013). However, all of the antimicrobials inhibited spore outgrowth to differing degrees, although the effect varied between strains. In addition, several muricholic acids and analogues of chenodeoxycholic acid have been suggested as potential inhibitors of spore germination *in vitro* (Sorg and Sonenshein 2010; Francis *et al.* 2013). Although the natural products did not have any effect on spore germination, the impact on the outgrowth of vegetative cells in addition to an inhibitory effect on spore formation could be an important approach in prevention of relapses of CDI. Moreover, prevention of outgrowth would inhibit toxin production from vegetative cells and reduce or prevent other subsequent effects (Allen *et al.* 2013). We have previously shown the significant inhibitory effect *in vitro* that some of these substances have on vegetative *C. difficile* (Roshan *et al.* 2017); however, additional studies

Table 5 Sporidicidal activity of selected natural products against *Clostridium difficile* isolates

Micro-organisms	Incubation time	Log reduction in viable spores										SDW	DM50 (% v v ⁻¹)
		Peppermint oil (% v v ⁻¹)					trans-Cinnamaldehyde (% v v ⁻¹)						
		16	8	4	2	1	16	8	4	2	1		
<i>Clostridium difficile</i> NCTC 13366	24 h	1.19 ± 0.12***	1.23 ± 0.05***	1.14 ± 0.08***	0.61 ± 0.10	0.51 ± 0.11	1.12 ± 0.30***	1.00 ± 0.11***	0.72 ± 0.09**	0.68 ± 0.08*	0.69 ± 0.05*	0.08 ± 0.11	0.07 ± 0.07
	48 h	1.67 ± 0.54***	1.18 ± 0.16***	1.17 ± 0.05***	1.05 ± 0.09***	0.80 ± 0.10	1.75 ± 0.41***	1.64 ± 0.21***	1.11 ± 0.10***	1.11 ± 0.23***	0.80 ± 0.23*	0.12 ± 0.07	0.08 ± 0.05
	7 d	1.92 ± 0.22***	1.76 ± 0.23***	1.26 ± 0.29***	1.21 ± 0.23***	0.84 ± 0.14*	2.10 ± 0.21***	1.80 ± 0.26***	1.46 ± 0.28***	1.34 ± 0.12***	0.80 ± 0.31*	0.12 ± 0.08	0.15 ± 0.06
<i>Clostridium difficile</i> ATCC 700057	24 h	1.21 ± 0.18***	1.07 ± 0.07***	0.94 ± 0.18***	0.63 ± 0.25*	0.57 ± 0.19	1.14 ± 0.24***	1.13 ± 0.21***	1.00 ± 0.13***	0.89 ± 0.26***	0.74 ± 0.11**	0.10 ± 0.09	0.09 ± 0.03
	48 h	1.72 ± 0.028***	1.55 ± 0.48***	1.68 ± 0.29***	1.25 ± 0.38***	0.88 ± 0.44	1.84 ± 0.24***	1.32 ± 0.23***	1.33 ± 0.29***	1.09 ± 0.23**	0.93 ± 0.18*	0.09 ± 0.01	0.06 ± 0.03
	7 d	1.94 ± 0.47***	1.67 ± 0.36***	1.27 ± 0.10**	1.43 ± 0.50***	1.09 ± 0.36*	2.15 ± 0.34***	1.70 ± 0.17***	1.48 ± 0.27***	0.98 ± 0.11*	0.99 ± 0.09*	0.08 ± 0.02	0.06 ± 0.01

SDW, sterile distilled water; DMSO, dimethyl sulfoxide; h, hour; d, day.
Statistical significance: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared to untreated controls (SDW and DMSO).

such as randomized controlled trials are required to investigate appropriate dosing and to confirm clinical utility.

Patients suffering from CDI with a prolonged stay in hospital shed large number of both vegetative cells and spores of *C. difficile* into their surroundings; consequently anything contaminated with the spores including surfaces, and hands of healthcare workers can become a vector of transmission (Pyrek 2013). Hence, it is critical to apply effective spore-killing treatments to reduce the rate of transmission. Among the five natural products tested for sporidicidal activity against *C. difficile*, peppermint oil (4% v v⁻¹) and *trans*-cinnamaldehyde (1% v v⁻¹) showed killing of spores for the selected *C. difficile* strains after 24 h. However, these concentrations resulted in log₁₀ reduction values of ≤1 after 24 h and higher concentrations of either product, and longer exposure times, were required for greater sporidicidal activity. Therefore, the findings from this assay may limit its practical use. In a study by Lawrence and Palombo (2009), all essential oils tested against *Bacillus subtilis* endospores showed some sporidicidal activity. Moreover, several previous studies have investigated the sporidicidal effect of various disinfectants against *C. difficile* endospores, but no published studies on the sporidicidal activity of natural products against *C. difficile* spores were found (Wheeldon *et al.* 2008; Speight *et al.* 2011; Vohra and Poxton 2011).

Endospores are resistant to antimicrobials and there is currently no Environmental Protection Agency-registered disinfectant available for inactivation of *C. difficile* spores. The recommendations for decontamination of spores are based on evidence available from the scientific literature (Pyrek 2013). Therefore, further studies to identify other alternatives with sporidicidal activity are essential. Many of the products tested in this study are already commercially available for human consumption. However, natural products should not always be assumed to be safe, and ingestion of some of these products in large quantities may result in adverse side effects such as toxicity or alterations in levels or composition of commensal flora (U.S. Department of Health and Human Services 2015). In this study, the products showing activity at concentrations that are safe for human consumption may be worthy of further investigation for activity *in vivo*. Although no chemical analysis of crude extracts was performed, the active components in some, such as allicin, *trans*-cinnamaldehyde, menthol and zingerone, were tested, and in some cases showed activity. However, it is also important to emphasize that the activity of natural products varies depending on the plant species they are derived from, and may also vary between different samples of the same plant. Further studies on chemical composition of these products will be of the importance and until such studies

are done, investigations of “natural products” should be interpreted with caution.

Nonetheless, our data showed that several natural products have inhibitory effects on sporulation and the vegetative outgrowth of *C. difficile* *in vitro*. The sporicidal activity obtained with two of these products, peppermint oil and *trans*-cinnamaldehyde, may not be practical as a long exposure time and high concentrations of those products were required to show substantial reduction in *C. difficile* spores. However, the inhibition of sporulation as shown in this study may be a powerful way to reduce infection transmission. In addition, inhibiting the conversion of spores to vegetative cells could contribute to reducing recurrence of CDI. Additional studies are required to further understand the effect and mechanism of action of natural products on different stages of the spore cycle of *C. difficile* and their potential effects *in vivo*.

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Conflict of Interest

No conflict of interest is declared.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The percentage of spores after 48 and 96 h growth in liquid TY broth containing $0.25 \times \text{MIC}$ of natural products or antibiotics determined by conventional spore recovery and microscopy.

Figure S2. Effect of natural products on germination and outgrowth of *Clostridium difficile* spores.

Figure S3. Effect of natural products on germination and outgrowth of *Clostridium difficile* spores.

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Effect of natural products on the production and activity of *Clostridium difficile* toxins *in vitro*

Niloufar Roshan¹, Thomas V. Riley^{1,2,3,4}, Daniel R. Knight³ & Katherine A. Hammer¹

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Clostridium difficile infection is a toxin-mediated disease of the colon. *C. difficile* virulence is primarily attributed to the production of toxin A and toxin B; thus this study was aimed to investigate the effect of a range of natural products on the production and activity of *C. difficile* toxins *in vitro*. Twenty-two natural products were investigated against four *C. difficile* strains. The activity of products against toxins was determined using Vero and HT-29 cells cytotoxicity and neutral red uptake assays. The indirect effect of products on toxin-mediated cytotoxicity was determined using the same cell lines. The effect of seven products on toxin production by *C. difficile* was determined using ELISA. Zingerone (0.3 mg/ml) protected both cell lines from *C. difficile* cytopathic effects, confirmed by the neutral red uptake assay ($P < 0.05$). Three *Leptospermum* honeys (4% w/v), fresh onion bulb extract (12.5% v/v) and *trans*-cinnamaldehyde (0.005% v/v) all reduced toxin production and activity significantly ($P \leq 0.023$). Garlic clove powder (4.7 mg/ml) only reduced toxin activity ($P \leq 0.047$). Overall, several natural products had activity against *C. difficile* toxins *in vitro* encouraging further investigation against *C. difficile* toxins *in vivo*.

Clostridium difficile infection (CDI) is one of the most important healthcare-associated infections worldwide and a major cause of morbidity and mortality in both the hospital and community settings¹. Typically, CDI occurs following the disruption of normal enteric flora, usually post-antimicrobial exposure, leading to the proliferation and germination of *C. difficile* spores into vegetative cells, resulting in toxin production in the intestine². Toxin A (TcdA, 308 kDa) and toxin B (TcdB, 270 kDa) belong to the large clostridial glucosylating family of toxins and are the major virulence factors of this species³. Despite similar enzymatic activities, TcdA and TcdB have different cellular receptors, *in vivo* potency and immunological response, and recent studies of isogenic toxin mutants in hamster and piglet models provided convincing evidence that toxin B alone is essential for CDI^{3,4}. Secretion of these potent toxins within the gastrointestinal tract causes actin disassembly, enterocyte apoptosis, the breakdown of epithelial tight junctions and an overall loss of epithelial integrity³. This pathophysiological cascade results in extensive colonic inflammation, epithelial tissue damage and a rapid loss of fluid into the intestinal lumen, manifesting as characteristic watery diarrhoea but may also develop into fatal sequelae including pseudomembranous and fulminant colitis^{3,4}. *In vitro* cellular apoptosis is seen clearly in the characteristic “cell rounding” phenotype or cytopathic effect (CPE). Some *C. difficile* lineages, most notably the hypervirulent NAP1/027 epidemic strain, also produce a third toxin known as binary toxin (CDT), however, there is still little known about the role of this toxin in virulence⁵.

For the last three decades, CDI has been managed with the conventional antimicrobials metronidazole (for mild to moderate CDI) and vancomycin (for severe CDI)⁶. Despite retaining good *in vitro* efficacy against *C. difficile*, several issues remain surrounding the use of these agents. Increasingly, they are associated with unacceptably high rates of CDI recurrence, reduced efficacy *in vitro* and minimal inhibitory concentration (MIC) creep, particularly with metronidazole, and with vancomycin, there is a risk of selection for acquired glycopeptide resistance in Gram-positive pathogens^{7–9}. Fidaxomicin became available for treatment of CDI in 2011. It has a

¹School of Biomedical Sciences (M504), The University of Western Australia, 35 Stirling Hwy, Crawley, Western Australia, 6009, Australia. ²Division of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, Western Australia, 6009, Australia. ³School of Veterinary & Life Sciences, Murdoch University, Murdoch, Western Australia, 6150, Australia. ⁴School of Medical & Health Sciences, Edith Cowan University, Joondalup, Western Australia, 6027, Australia. Correspondence and requests for materials should be addressed to K.A.H. (email: katherine.hammer@uwa.edu.au)

narrower spectrum of activity compared to vancomycin and metronidazole, and thus may have less impact on gut microbiota. However, its high cost compared to either metronidazole or vancomycin has restricted its use¹⁰.

Medicinal plants remain the primary source of treatment for several diseases in rural areas of many developing countries¹¹. Plant-derived compounds are considered by some consumers as a safer, less toxic and more environmentally-friendly option compared to conventional therapies. They are typically multi-component in nature and the components contain different functional groups. As such, their antimicrobial activity often relates to multiple mechanisms; hence, unlike conventional antimicrobials, organisms are less likely to develop resistance¹². We have shown recently that several natural products have antimicrobial activity against *C. difficile* *in vitro*¹³. Given that CDI is a toxin-mediated disease, the purpose of the present study was to investigate the effect of those previously tested natural products, and corresponding pure substances, on toxin production and activity in *C. difficile* *in vitro*. The anti-toxin activity of natural products and their impact on toxin-mediated cytotoxicity were assessed using tissue culture and cytotoxicity assays. ELISA was performed to determine the effect of these products on the production of *C. difficile* toxins.

Results

Antimicrobial susceptibility assay. The MICs of natural products (Table 1)¹⁴ and antimicrobial controls for the four strains of *C. difficile* have previously been published¹⁵ and are shown in Table S1. The only exception was the two extra *Leptospermum* honeys A and B as listed in Table 1. Both honeys A and B showed an MIC of 16% v/v, which was two-fold lower than previously tested *Leptospermum* honey C.

Toxin protection assay. The supernatants from the three toxigenic strains demonstrated CPE in both Vero and HT-29 cells. However, only minor CPE was observed with *C. difficile* ATCC 43598 and HT-29 cells. This was shown as cell rounding, membrane blebbing and loss of adhesion. The toxin titres for each of the three toxigenic *C. difficile* strains causing approximately 90% CPE were determined over three independent experiments. A modal toxin titre was selected for each strain (Table 2). The highest concentrations of each antimicrobial agent showing no effect on cells are shown in Table S2. In addition, the pH of the growth medium with and without the addition of each compound was measured to determine whether there was a deviation in pH that could interfere in the assay. No significant deviation was observed. When culture filtrates were incubated for 2 h with antimicrobial agents, only zingerone protected Vero cells against any cytotoxic effect visually. This was confirmed by a neutral red uptake assay ($P \leq 0.007$) (Figs 1, S1 and S2). Also, when Vero cells were pre-incubated with zingerone for 2 h prior to the addition of culture filtrates, a similar protective effect was observed. However, when zingerone and culture filtrates were transferred to the wells containing Vero cell monolayers together, and with no prior incubation, no protection was observed. Zingerone showed a similar protective effect against toxin activity on HT-29 cell monolayers (Figs 2, 3 and S3).

Indirect effect of natural products on toxin-mediated cytotoxicity. The effect of all products on toxin-mediated cytotoxicity on Vero cells was examined for *C. difficile* NCTC 13366 and R44116 (Table S3), and products showing a reduction in CPE were further tested against *C. difficile* ATCC 43598. Exposure to six of the 22 natural products significantly reduced cytotoxicity with Vero cells compared to untreated culture filtrate of the three toxigenic strains of *C. difficile* ($P \leq 0.047$) (Fig. 4). The cytotoxicity titres for *C. difficile* cultures incubated for 48 h with $0.5 \times$ and $0.25 \times$ MICs of fresh onion bulb extract, garlic clove powder, *trans*-cinnamaldehyde and the three *Leptospermum* honeys were reduced by $\geq 70\%$ compared to the untreated controls. Similar findings were observed for HT-29 cells ($P \leq 0.001$), with the exception of garlic powder at $0.25 \times$ MIC which did not show any reduction in CPE.

Effect of treatments on *C. difficile* toxin production. Seven of the 22 products that showed activity in either direct or indirect toxin activity assays were selected to determine their effect on toxin production by the three toxigenic *C. difficile* strains using ELISA. Toxin production for *C. difficile* strains in the presence of treatments relative to untreated cultures is shown in Fig. 5. Fresh onion bulb extract, *trans*-cinnamaldehyde and the three *Leptospermum* honeys significantly reduced toxin production by all three toxigenic strains compared to the untreated controls ($P < 0.001$). At 48 h, these products inhibited toxin production by $\geq 40\%$ in the toxigenic strains.

Discussion

Toxins are the main virulence factors of *C. difficile*, which initiates gastrointestinal disease by causing inflammation and mucosal damage in the intestine¹⁵. Therefore, investigating toxin-active therapeutic agents with low risk of developing antimicrobial resistance is of great interest in either treating or preventing CDI⁸. In this study, the effect of a range of natural products, and the corresponding pure substance, on toxin activity of *C. difficile* was examined using Vero and HT-29 cells. Similar results were observed with both cell lines. The toxin protection study showed that the ginger component zingerone protected cells against the effect of *C. difficile* toxin. In this assay, protection against toxin activity was observed when culture filtrates and zingerone were incubated together for 2 h prior to being added to the cells, as well as when the cells were pre-incubated with zingerone prior to addition of culture filtrates. However, no significant protection was observed when toxin filtrates and zingerone were added simultaneously to wells containing either Vero or HT-29 cells. Hence, it is likely that zingerone provides protection against the action of *C. difficile* toxin by blocking either the toxin-binding sites on toxin molecules or the host cell receptors. In an earlier study by Chen *et al.*, the effect of ginger and its bioactive components on inhibition of enterotoxigenic *Escherichia coli* heat-labile enterotoxin (LT)-induced diarrhea in mice was investigated. Ginger extract inhibited fluid accumulation in the ileal loops of mice by blocking the binding of LT to G_{M1} cell-surface receptor. Further investigation of biologically-active components showed that zingerone was the active constituent responsible for the anti-diarrhoeal effect of ginger¹⁶.

Product	Concentration	Ingredient information	Supplier
Raw products			
Fresh garlic bulb	1 ml/ml	None	—
Fresh onion bulb	1 ml/ml	None	—
Fresh ginger rhizome	1 ml/ml	None	—
<i>Leptospermum</i> honey (MGO 514+) (A)	100% pure Manuka honey	None	Barnes Naturals, Richlands, QLD, Australia
<i>Leptospermum</i> honey (MGO 263+) (B)	100% pure Manuka honey	None	Barnes Naturals
<i>Leptospermum</i> honey (MGO 263+) (C)	100% pure Manuka honey	None	Barnes Naturals
Garlic clove powder	1 mg/mg	None	Spencers, Fremantle, WA, Australia
Ginger rhizome powder	1 mg/mg	None	Spencers
Cinnamon root powder	1 mg/mg	None	Spencers
Turmeric root powder	1 mg/mg	None	Spencers
Processed products			
Garlic tablet	Garlic (<i>Allium sativum</i>) extract equivalent to dry bulb 10,000 mg	No added yeast, starch, gluten, lactose, sugar, artificial colours or flavours, artificial sweeteners or preservatives, dairy products or animal-derived products.	Nature's Own, Virginia, QLD, Australia
Ginger tablet	1000 mg	No added yeast, starch, gluten, lactose, sugar, artificial colours or flavours, artificial sweeteners or preservatives, dairy products or animal-derived products.	Nature's Own
Cinnamon tablet	Cinnamon (<i>Cinnamomum cassia</i>) stem bark inner powder 1000 mg	No added lactose, egg, gluten, soy, yeast, artificial colours and artificial flavours.	Swisse, Collingwood, VIC, Australia
Turmeric tablet	1000 mg	Turmeric powder 77%, cellulose microcrystalline, povidone, silica colloidal anhydrous, magnesium stearate, croscarmellose sodium.	Nature's Way, Warriewood, NSW, Australia
Processed products			
Artichoke capsule	Herbal extract equivalent to <i>Cynara scolymus</i> fresh leaf 60000 mg	No added lactose, starch, added sugars and salt, artificial colourings and flavourings or preservatives.	Nature's Sunshine, Baulkham hills, NSW, Australia
Coconut oil capsule	100% coconut oil	Capsule shell (gelatin, glycerol, water)	Nature's Way
Peppermint oil	100% pure, <i>Mentha X piperita</i> leaf 1 ml/ml	None	Oil Garden, Springvale, VIC, Australia
Aloe vera gel	99% pure <i>Aloe Barbadensis</i>	Allantoin (found in Comfrey root), carbomer, disodium edetate, sodium hydroxyl methylglycinate, grapefruit seed extract	Plunkett's, Warriewood, NSW, Australia
Pure compounds			
Allicin	180 mg allicin extract (garlic bulb)	Vegetarian capsule (hypromellose, water), maltodextrin, gum acacia, Made without corn, wheat, gluten, yeast, soy, sugar, dairy, artificial colors or flavors	Vcaps, Chicago, IL, USA.
<i>trans</i> -Cinnamaldehyde	Purity >98.5%	None	Sigma Aldrich, Saint Louis, MO, USA
Menthol	Purity >98.5%	None	Sigma Aldrich
Zingerone	Purity >96%	None	Sigma Aldrich

Table 1. Range of natural products used in this study. MGO, methylglyoxal. (Table extracted from Roshan *et al.* 2018).

With the indirect toxin-mediated cytotoxicity assay and ELISAs, several products such as the three *Leptospermum* honeys, fresh onion bulb extract and *trans*-cinnamaldehyde at 0.5 and 0.25 $\times$ MIC, showed a reduction in both toxin production and activity. Garlic clove powder at 0.5 and 0.25 $\times$ MIC showed a reduction in toxin activity with Vero cells and at 0.5 $\times$ MIC with HT-29 cells. Also, the ELISAs did not indicate any significant decrease in toxin production with garlic powder compared to the untreated controls. Previously, *trans*-cinnamaldehyde (0.38 mM; 0.05 mg/mL) reduced both *C. difficile* toxin production and activity *in vitro*¹⁷. In a study by Upadhyay *et al.*, *trans*-cinnamaldehyde (0.5 mM) down-regulated *hly* and *prfA* genes coding for toxin production and a transcriptional regulator in *Listeria monocytogenes*¹⁸. The activity of several antimicrobials such as fidaxomicin, its major metabolite (OP-1118), metronidazole and vancomycin against *C. difficile* toxin has been investigated previously¹⁹. That study showed that both fidaxomicin and OP-1118 at 0.25 $\times$ MIC inhibited toxin production by $\geq 60\%$ following 1 week of culture. Also, a near complete inhibition of toxin gene expression was observed with both fidaxomicin and OP-1118 at 2 and 2.5 $\times$ MIC, respectively. No inhibitory effect on *C. difficile* toxin production or gene expression was reported for either metronidazole or vancomycin¹⁹. In our study, fidaxomicin was used as an antimicrobial control and showed a reduction in both *C. difficile* toxin production and activity.

Notwithstanding the protective effect of zingerone against *C. difficile* toxin observed in the toxin protection assay, zingerone did not show any effect in either the indirect toxin-mediated cytotoxicity assay or toxin production assays. Theoretically, zingerone has a relatively rapid bactericidal action and its activity may degrade over time, although there are no published literature to support this hypothesis. In indirect toxin-mediated cytotoxicity

<i>C. difficile</i> strain	Modal toxin titre (range)	
	Vero cells	HT-29 cells
NCTC 13366	1:524,288 (1:524,288 – 1:2,097,152)	1:262,144 (131,072–524,288)
R11446	1:8,192 (1:2,048 – 1:8,192)	1:8,192 (4,096–8,16,384)
ATCC 43598	1:512 (1:512 – 1:1,024)	1:8 (1:8–1:32)

Table 2. *C. difficile* toxin titre required to achieve 90% CPE on Vero and HT-29 cells after 24 h incubation at 37 °C with 5% CO₂.

and toxin production assays, treatments were not exposed directly to the toxin and it would have had taken some time for *C. difficile* to initiate toxin production. By the time the cells had started to produce toxin, the activity of zingerone may have been minimal. Moreover, since gene expression for toxin production occurs during the late log-phase, the addition of the compound during this stage could determine the effect of zingerone on toxin gene expression. This will be studied in a series of experiments in the future.

Food-grade and plant-derived compounds are used commonly among people who practice self-health care¹⁰. Many consumers turn to natural products assuming that natural is synonymous with safe. This concept is not necessarily true as side effects are expected with some²⁰. Some natural products such as *trans*-cinnamaldehyde are toxic to humans if consumed in large quantities²¹. However, many natural products, including those used in this study, have “generally recognised as safe” (GRAS) status with the US Food and Drug Administration. This means that when consumed in moderate amounts they are unlikely to pose a health risk²¹. In addition, further studies on the chemical composition of these products, as distinct from pure substances, will be of importance. Until such studies are done, the results from investigations of “natural products” should be interpreted with caution.

Conclusions

Overall, this study highlights the activity of a number of food-grade and plant-derived products against *C. difficile* toxins *in vitro*. These results suggest that several natural products may have the potential to be considered as either alternative or complementary treatment options for CDI. Applying anti-virulence strategies such as disruption of toxin production and toxin-mediated pathology in patients with CDI has the potential to be an effective approach in either controlling or treating infection. However, further studies are required to investigate the mechanistic basis for the anti-toxin activity observed as well as to determine their potential benefits *in vivo*.

Material and Methods

Bacterial strains. The following four *Clostridium difficile* strains were used in this study: non-toxicogenic *C. difficile* ATCC 700057 (A⁺B⁺CDT⁺), *C. difficile* NCTC 13366 (PCR ribotype (RT) 027, A⁺B⁺CDT⁺), *C. difficile* R11446 (RT 014, A⁺B⁺CDT⁺, clinical isolate) and *C. difficile* ATCC 43598 (RT 017, A⁺B⁺CDT⁺). Strains were obtained from the School of Biomedical Sciences at The University of Western Australia and PathWest Laboratory Medicine, WA.

Antimicrobial agents. Twenty-two natural products were selected for investigation based on historical evidence, their current popularity, and feasibility (Table 1). The products were categorized into three broad groups; raw products usually consumed as food, processed products taken as health supplements and the active pure compounds found in these products. The raw products (n = 10) were purchased from a supermarket in Perth, Western Australia (WA) (Table 1) while the *Leptospermum* honeys were kindly provided by Capilano Company, Bayswater, WA. Processed and pure products (n = 12) were purchased from pharmacies and health food stores in Perth, WA, or from the manufacturers (Table 1). The products were prepared for testing as previously described¹³. Fidaxomicin and vancomycin were used as antimicrobial controls in susceptibility testing and fidaxomicin was used as an effective-antimicrobial control in tissue culture and ELISA assays. Stock solutions of products in the form of powder or oil were prepared in 20% dimethyl sulfoxide (DMSO). Further dilutions were performed in sterile distilled water (SDW) for all products. Prior to testing, raw products were filtered through a 0.22 µm-pore size Millipore filter to remove any microbial contamination, however, the processed products were not filtered as they were assumed to have had gone through a sterilization process during manufacturing.

Determination of minimum inhibitory concentration (MIC). Minimum inhibitory concentrations (MIC) for natural products and comparator antimicrobial agents were determined by the broth microdilution method as previously described¹³. Doubling dilutions of 20% DMSO were prepared and tested in parallel (solvent control), and fidaxomicin and vancomycin were included as antibiotic controls. The previously determined MICs for these products against the stationary phase *C. difficile* were used in this study.

Toxin protection assay. *Tissue culture.* The toxin protection assay was performed as described previously⁸ with slight modification. The Vero (African green monkey kidney) and HT-29 (human colon carcinoma) cells were passaged in a 96-multiwell plate and incubated for 24 h prior to use. To prepare confluent monolayers, Vero and HT-29 cells were dispensed into 96-well trays at 6 × 10⁴ cells/well and 5 × 10⁵ cells/well, respectively. Vero cells were passaged and maintained in Dulbecco’s Modified Eagles Medium (DMEM). DMEM supplemented with 10% (DMEM-GM) and 2% foetal bovine serum (DMEM-MM) were used as growth and maintenance medium, respectively. Both DMEM-GM and DMEM-MM also contained 200 µg/ml vancomycin and 100 µg/ml streptomycin sulfate. HT-29 was passaged and maintained as above, except that the medium also contained glucose (6 g/l).

Figure 1. Protection from cytopathic effect on Vero cells using microscopy (*C. difficile* NCTC 13366 culture filtrate and zingerone were incubated at 37 °C for 2 h prior to being added to Vero cell monolayers). (A) No culture filtrate; (B) culture filtrate only; (C) zingerone (1.2 mg/ml); (D) zingerone (0.6 mg/ml); (E) zingerone (0.3 mg/ml); Light microscopy $\times 40$, Scale: 50 μ m.

Figure 2. Protection from cytopathic effect on HT-29 cells using microscopy (*C. difficile* NCTC 13366 culture filtrate and zingerone were incubated at 37 °C for 2 h prior to being added to HT-29 cell monolayers). (A) No culture filtrate; (B) culture filtrate only; (C) zingerone (1.2 mg/ml); (D) zingerone (0.6 mg/ml); (E) zingerone (0.3 mg/ml); Light microscopy $\times 40$, Scale: 50 μ m.

Figure 3. Cell viability determined by neutral red uptake assay. (Culture filtrates and treatments were incubated 2 h prior to being added to the cells); (A) Vero cells; (B) HT-29 cells; Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to culture filtrate control.

Culture filtrate preparation. *C. difficile* strains were cultured on blood agar for 48 h, from which suspensions equivalent in turbidity to a 2 McFarland standard were prepared in 0.85% saline. A volume of 100 μ l of each suspension was inoculated into a 10 ml pre-reduced brain heart infusion broth (BHIB). Following 5 h of anaerobic incubation, the broth cultures were aseptically sealed and incubated for a further 5 days at 37 °C in a Thermocube incubator (Bioline) with shaking at 120 revolutions per minute (rpm). After 5 days of incubation, cultures were centrifuged at 4,000 $\times g$ for 5 min and the supernatant containing toxin was passed through a 0.22 μ m-pore size Millipore filter to remove cells and debris.

Toxin titration. Serial two-fold dilutions of culture filtrate in DMEM-MM were prepared with a total volume of 100 μ l per well and were added to the cells. The 90% cytotoxicity titre was determined visually using an inverted microscope (Olympus Corporation, Tokyo, Japan) at $\times 400$ magnification. All titrations were performed three times.

Figure 4. Indirect effect of treatments on *C. difficile* cytotoxicity on (A) Vero cells and (B) HT-29 cells. Concentrations of agents used in the assay: Fresh onion bulb extract (25% and 12.5% v/v); Garlic clove powder (4.7 and 2.3 mg/ml); *Leptospermum* honey (A), (B) and (C) (8% and 4% v/v); trans-Cinnamaldehyde (0.01% and 0.005 v/v); Zingerone (4.7 and 2.3 mg/ml); Fidaxomicin (0.06 µg/ml). Statistical significance: $^{\Delta}P < 0.05$, $^{\circ}P < 0.01$, $^{*}P < 0.001$ compared to controls; *LH, *Leptospermum* honey; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

Preparation of natural products. Natural products were prepared as described above. Serial two-fold dilutions of natural products, fidaxomicin and DMSO were prepared in DMEM-MM and 100 µl volumes of each were added to Vero cell monolayers. The highest concentrations showing no visible effect on cell monolayers after 24 h incubation was used in the subsequent assay.

Incubation of products and culture filtrates. The highest concentrations of all products with no visible effect on cell monolayers, in addition to 2-fold and 4-fold lower concentrations, were used in this assay. Those products that showed protection against toxin on Vero cells were also tested on HT-29 cells. Supernatants from the three toxigenic *C. difficile* strains were diluted to 4-fold higher than the minimum dilution required to achieve 90% cell rounding to account for the possible variation and dilution factor. The culture filtrates and products were both diluted in DMEM-MM. To evaluate whether natural products reduced the activity of free toxin, diluted antimicrobial agents and supernatants were mixed at a ratio of 1:1 and incubated at 37 °C for 2 h. Aliquots of 100 µl of 1:1 antimicrobial agents: culture filtrates were added to 96-well microtiter plates containing Vero cell monolayers and incubated for 24 h at 37 °C in a CO₂ incubator (Thermo Fisher Scientific, Waltham, MA USA) containing 5% CO₂. Protection from CPE was evaluated with an inverted microscope and was verified with a neutral red uptake assay (see below).

Culture filtrates from non-toxicogenic *C. difficile* ATCC 700057 and the three other toxigenic strains without the addition of antimicrobial agents were used as controls, with the three toxigenic strains showing a CPE but not the non-toxicogenic strain. The assay was repeated on three different occasions. If protection was achieved by any of the products, they were further investigated to assess their likely mode of action, such as blocking the binding sites or inactivating the toxin. To investigate whether that product blocked cell binding sites, the cell monolayers were pre-incubated for 2 h with treatments at 2-fold higher than the desired concentrations at 37 °C with 5% CO₂, followed by the addition of an equivalent amount of *C. difficile* culture filtrate. Lastly, to evaluate whether protection occurred immediately or pre-incubation was required, diluted antimicrobial agents and culture filtrates were mixed at a ratio of 1:1 and 100 µl of the mixture was immediately transferred to the wells containing cell monolayers.

Neutral red uptake assay. A neutral red uptake assay was performed to determine cell viability and to confirm the visual perception of protection from CPE²¹. Following the visual evaluation of results after 24 h of incubation, the contents from each well were removed and a 100 µl aliquot of 40 µg/ml neutral red solution was added. Plates were incubated for 3 h at 37 °C with 5% CO₂, then washed with 150 µl of phosphate buffered saline

Figure 5. Effect of treatments on *C. difficile* toxin production. Concentrations of agents used in the assay: Fresh onion bulb extract (25% and 12.5% v/v); Garlic clove powder (4.7 and 2.3 mg/ml); *Leptospermum* honey (A,B) and (C) (8% and 4% v/v); *trans*-Cinnamaldehyde (0.01% and 0.005 v/v); Zingerone (4.7 and 2.3 mg/ml); Fidaxomicin (0.06 µg/ml). Statistical significance: ^A $P < 0.05$, ^O $P < 0.01$, ^{*} $P < 0.001$ compared to controls; *LH, *Leptospermum* honey; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

(PBS pH 7.3). To elute the stain, 150 µl of neutral red destain solution was added to each well and the plates were placed on a shaker at 120 rpm for 10 min. The optical density of each well was measured at 540 nm using an xMark™ microplate spectrophotometer (Bio-Rad). Wells without cells served as blanks. The percentage of viable cells in wells containing treated and untreated culture filtrates was calculated relative to the untreated control cells, which were assumed to be 100% viable.

Indirect toxin-mediated cytotoxicity assay. The indirect effect of all 22 products on the activity of *C. difficile* toxins was determined using the Vero cell cytotoxicity assay as described previously²². Those products showing any effect against toxin on Vero cells were further tested with HT-29 cells. Briefly, a bacterial suspension of 1×10^6 cfu/ml was prepared for each strain in pre-reduced BHIB containing $0.5 \times \text{MIC}$ and $0.25 \times \text{MIC}$ of treatments. The final volume for each bacterial suspension was 5 ml after addition of treatments. After incubating the cultures for 48 h anaerobically at 35 °C, they were centrifuged at 4,000 g for 10 min and the supernatant filtered through a 0.22 µm pore-size membrane filter. The culture filtrates were diluted 1:100 prior to being added to the cell monolayer plates to reduce the gross effect of products on cells. The diluted culture filtrates were further serially diluted 1:2 in 96-well microtiter plates containing cell monolayers using DMEM-MM. The plates were incubated at 37 °C in 5% CO₂ for 48 h and examined under an inverted microscope. The reciprocal of the highest dilution causing 90% cell rounding was expressed as the cytotoxicity titre. All the products were tested against *C. difficile* NCTC 13366 and *C. difficile* R11446 using Vero cells and those products showing a reduction in cytotoxicity titre were further tested against *C. difficile* ATCC 43598. Only *C. difficile* NCTC 13366 and *C. difficile* R11446 were used for the HT-29 cell line, as just a minor CPE was observed with *C. difficile* ATCC 43598 with those cells. Fidaxomicin was used as a positive control (reducing CPE) and culture filtrate from *C. difficile* ATCC 700057 was used as a negative control (showing no CPE) in this assay.

Effect of products on toxin production. The amount of toxin in cultures grown in the presence of $0.5 \times \text{MIC}$ and $0.25 \times \text{MIC}$ of treatments showing activity in either toxin activity assays was determined relative to the amount of toxin in control cultures (grown without antimicrobials) using the *C. difficile* TOX A/B II™ ELISA kit (TechLab®). Also, fidaxomicin was included as an antimicrobial control. The culture supernatant from

C. difficile strains was prepared and filtered as described earlier. The culture filtrates were diluted and tested according to the manufacturer's instructions. The spectrophotometer was blanked against air at a wavelength of 620 nm and the optical density (OD) was measured at 450 nm. Relative toxin production was expressed as a percentage of OD₄₅₀ for treated culture filtrates over OD₄₅₀ of untreated culture filtrates.

Statistical analysis. All assays were repeated at least three times. A modal value was determined for MIC values obtained by the broth microdilution assay and for the cytotoxicity titres in the indirect cytotoxicity assay. The mean and standard deviation (SD) were calculated for the percentage of viable cells in the neutral red uptake assay and for toxin production in ELISA assays. Statistical differences between three or more sets of data were analysed using GraphPad Prism v.7 software using one-way analysis of variance (ANOVA) and nonparametric technique, followed by Tukey's multiple comparison post-test if the *P* value was significant. Moreover, the Student's 2-tailed *t*-test assuming unequal variance was used to determine whether there was a significant difference between two sets of data. Toxin titres in the indirect toxin study were converted to log₂ values and titre reciprocals that were lower than 100 were converted to a lower titre reciprocal of 50 to enable analysis. *P* values of <0.05 were considered significant.

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Author Contributions

K.A.H. and T.V.R. conceived the study. N.R. designed and performed the experiments. All authors contributed to drafting the manuscript. All authors reviewed and approved the manuscript.

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ORIGINAL ARTICLE

Natural products show diverse mechanisms of action against *Clostridium difficile*

N. Roshan¹ , T.V. Riley^{1,2,3,4}, D.R. Knight³, J.H. Steer¹ and K.A. Hammer¹

¹ School of Biomedical Sciences (M504), The University of Western Australia, Crawley, WA, Australia

² Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia

³ School of Veterinary & Life Sciences, Murdoch University, Murdoch, WA, Australia

⁴ School of Medical & Health Sciences, Edith Cowan University, Joondalup, WA, Australia

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Correspondence

Katherine A. Hammer, School of Biomedical Sciences (M504), The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia.
E-mail: katherine.hammer@uwa.edu.au

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Abstract

Aims: To investigate the mechanisms of action of natural products with bactericidal (cinnamon root powder, peppermint oil, *trans*-cinnamaldehyde, menthol and zingerone) or bacteriostatic (fresh garlic bulb extract, garlic clove powder, *Leptospermum* honey and allicin) activity against two *Clostridium difficile* strains.

Methods and Results: Bactericidal products significantly reduced intracellular ATP after 1 h ($P \leq 0.01$), quantified using the BacTiter-Glo reagent, and damaged the cell membrane, shown by the leakage of both 260-nm-absorbing materials and protein, and the uptake of propidium iodide. Bacteriolysis was not observed, determined by measuring optical density of treated cell suspensions at 620-nm. The effect of three bacteriostatic products on protein synthesis was quantified using an *Escherichia coli* S30 extract system, with *Leptospermum* honey (16% w/v) showing significant inhibition ($P < 0.01$). Lastly, no products showed elevated minimum inhibitory concentrations against antimicrobial-resistant *C. difficile*, determined by broth microdilution.

Conclusions: Cytoplasmic membrane damage was identified as a mechanism of action that may contribute to the activity of several natural products against *C. difficile*.

Significance and Impact of the Study: This study describes the possible mechanisms of action of natural products against *C. difficile*, yet the efficacy *in vivo* to be determined.

Introduction

Natural products derived from food spices and other plant extracts have great antimicrobial potential against drug-resistant micro-organisms and, unlike conventional antimicrobials, multicomponent products are less susceptible to the development of antimicrobial resistance (AMR) due to the multiple mechanisms of action (Rossiter *et al.* 2017). The antimicrobial activity of these products often relates to their multicomponent nature, with each component having a different functional group and a potentially differing mode of action. Medicinal plants, including a range of food spices, are the primary source of treatment for many diseases in

rural areas of developing countries (Num and Useh 2014). Understanding the underlying mechanism (or mechanisms) of action of plant extracts can assist with the use of the extracts or derived compounds in a medical setting or in an environmental application (Tomlinson and Palombo 2005).

A range of spices and plant extracts has been used historically to treat diarrhoea and other gastrointestinal complaints (Num and Useh 2014). Whilst gastrointestinal disease can be caused by a diverse range of microbes, this study focuses on *Clostridium difficile*, a Gram-positive, anaerobic, spore-forming bacterium. It is an important causative agent of not only healthcare-associated diarrhoea but also, increasingly identified in patients with no

hospital contact (Banawas 2018). Severity of the disease can range from self-limiting diarrhoea to more severe manifestations such as pseudomembranous colitis and toxic megacolon (Riley 2010; Alam *et al.* 2015). *Clostridium difficile* causes nearly half a million infections each year in the USA, and approximately 29 000 patients die shortly after initial diagnosis (Heinlen and Ballard 2010). The average yearly number of hospitalizations as a result of *C. difficile* infection (CDI) is estimated to be over a quarter of a million per year in the USA alone (Heinlen and Ballard 2010; Bouza 2012). It is estimated that healthcare costs in excess of US\$3600 are used per case of CDI, which results in a cumulative cost of more than US\$1 billion in the USA per annum (Heinlen and Ballard 2010; Bouza 2012). For the last 30 years, CDI has been managed with the conventional antimicrobials metronidazole and vancomycin, for treating mild to moderate CDI, and severe CDI, respectively (Trubiano *et al.* 2016). However, several issues are associated with the use of these agents, including a high recurrence rate of 20–25%, reduced *in vitro* efficacy and minimum inhibitory concentration (MIC) creep, particularly with resistance in Gram-positive pathogens (Pépin *et al.* 2005; Baines and Wilcox 2015). In 2011, fidaxomicin was approved as a new treatment for CDI (Roshan *et al.* 2018b). This antimicrobial acts by inhibiting RNA polymerase during transcription in prokaryotes and has a narrower spectrum of activity compared to vancomycin and metronidazole. However, high cost compared to either metronidazole or vancomycin has restricted its use (Roshan *et al.* 2018b).

We have recently shown the antimicrobial activity of several natural products, spice extracts and components against *C. difficile* vegetative cells (Roshan *et al.* 2017) and on several different stages of *C. difficile* spore formation and germination *in vitro* (Roshan *et al.* 2018a). The purpose of the present study was to expand on these previous studies and to investigate the mechanism of action of nine natural products or components with promising activity. These included cinnamon root, peppermint oil, *trans*-cinnamaldehyde, menthol, zingerone, allicin, fresh garlic bulb extract, garlic clove powder and *Leptospermum* honey with methylglyoxal (MGO) of 514⁺, which we had previously shown to have activity against *C. difficile* *in vitro*. The time-kill kinetics for the selected natural products, in addition to their effect on ATP biosynthesis and the membrane integrity of *C. difficile*, were determined. The inhibitory effect of products on prokaryotic translation/protein synthesis was also assessed. Lastly, the possibility of antimicrobial cross-resistance was investigated by determining the effect of natural products against a panel of *C. difficile* strains previously characterized for AMR phenotype and associated genotype.

Materials and methods

Bacterial strains and sequence data

The two *C. difficile* reference strains used in the investigation were *C. difficile* ATCC 700057 (nontoxigenic; A[−]B[−]CDT[−]) and *C. difficile* NCTC 13366 (PCR ribotype 027, A⁺B⁺CDT⁺). The clinical isolates used in this study, along with their phenotypic and genotypic AMR profiles, are listed in Table S1. Strains were obtained from the School of Biomedical Sciences at The University of Western Australia and PathWest Laboratory Medicine (WA). To study cross-resistance, whole-genome sequencing and *in silico* AMR genotyping of these strains was performed, as previously described (Knight *et al.* 2016).

Antimicrobial agents

The nine natural products/components were prepared as previously described (Roshan *et al.* 2017, 2018a,b). In this study, the products/components with either bacteriostatic ($n = 4$) or bactericidal ($n = 5$) activity against *C. difficile* were investigated further for the possible mechanisms of action. Triton X-100 and vancomycin were used as positive controls in cell lysis assays, while nisin and vancomycin were the controls in the alterations of cell permeability and ATP-leakage assays. To investigate the inhibitory effects on protein synthesis, streptomycin sulphate and tetracycline were used as positive antimicrobial controls (inhibiting protein synthesis).

Determination of minimum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentrations and minimum bactericidal concentration (MBCs) for natural products/components and comparator antimicrobial agents against both stationary- and log-phase *C. difficile* were determined by the broth microdilution method (Roshan *et al.* 2017, 2018a,b).

Time-kill assay

Time-kill kinetics were evaluated against both log-phase and stationary-phase cultures as described by Wu *et al.* (2013) with slight modifications. The inocula for log-phase and stationary-phase *C. difficile* were prepared as described previously (Roshan *et al.* 2017, 2018a,b) except that the cell concentration was adjusted to be equivalent to 1×10^8 CFU per ml. Brucella broth supplemented with 5 $\mu\text{g ml}^{-1}$ haemin, 1 $\mu\text{g ml}^{-1}$ vitamin K₁, 5% v/v laked sheep blood (supplemented Brucella Broth; SBB)

was used for this experiment. Cultures in prereduced SBB were exposed to 1, 2, 4 and 8× MIC of each product/component with a final inoculum concentration of $\approx 1 \times 10^7$ CFU per ml. The final volume for each bacterial suspension was 5 ml after the addition of treatments. Samples were taken at 0, 1, 2, 4, 6 and 24 h and viable counts were performed by serially diluting samples 10-fold in prereduced 0.85% saline and spot-inoculating 20 μ l volumes in duplicate from each dilution onto prereduced supplemented Brucella agar. The number of surviving organisms was determined after 24–48 h incubation at 35°C in an A35 anaerobic chamber (Don Whitely Scientific Ltd, Shipley, West Yorkshire, UK). The limit of detection was 5×10^2 CFU per ml. Both sterile distilled water (SDW) and the highest concentration of dimethyl sulfoxide (DMSO) used in the experiment were included as controls.

ATP-leakage assay

ATP leakage was determined according to the methods of Wu *et al.* (2013). In brief, log-phase cultures of *C. difficile* NCTC 13366 (12 h, OD₆₀₀ $\approx$ 0.5) were prepared in prereduced SBB (Roshan *et al.* 2018a,b). Cultures were exposed to 1, 2, 4 and 8× MIC of cinnamon root powder, peppermint oil, *trans*-cinnamaldehyde, menthol or zingerone at an inoculum concentration of $\approx 1 \times 10^7$ CFU per ml in a volume of 5 ml and then incubated in the anaerobic chamber. After 1, 2 and 6 h of treatment, 1 ml samples were removed and centrifuged at 10 000 g for 10 min. Supernatants were collected and cell pellets were resuspended in prereduced brain heart infusion broth (BHIB). The ATP in resuspended pellets (intracellular) and supernatants (extracellular) was measured using a BacTiter-Glo kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The luminescence was detected using a POLARstar Optima Plate Reader (BMG LAB-TECH, Pretoria, South Africa). ATP levels were expressed as a percentage of untreated controls (SDW) and the mean and standard deviations (SD) from three independent experiments were determined.

Bacteriolysis assay

The bacteriolysis assay was performed as described previously (Carson *et al.* 2002) with slight modifications. The growth from 24 to 36 h stationary-phase cultures on blood agar was suspended in prereduced phosphate buffered saline (PBS) (pH 7.3). The suspensions contained treatments at *supra*-MIC concentrations (Table S2) with a final inoculum concentration of $\approx 1 \times 10^8$ CFU per ml. The final volume for each bacterial suspension was 5 ml after the addition of treatments. The suspensions were mixed

immediately on a vortex mixer, diluted 1 in 10 and the OD₆₂₀ was measured. The treatments and controls were incubated anaerobically at 35°C and additional samples were taken at 1, 2 and 6 h. The absorbance of diluted samples in triplicate was read against the appropriate blanks (corresponding dilutions of test agents prepared as above but without inoculum). Triton X-100 was used as a positive control, while vancomycin was used as a negative control. SDW and the highest concentration of DMSO used in the experiment were also included as controls. The results were expressed relative to time zero by dividing the OD₆₂₀ at each time point by the OD₆₂₀ at time 0. The assay was repeated three times and the mean and SD determined.

Assays to determine alterations in cell permeability

Bacterial suspensions were prepared as described above for the bacteriolysis assay and exposed to various concentrations of treatments including 1×, 2×, 4× and 8× MIC. The final volume for each bacterial suspension was 5 ml after the addition of treatments. For all assays, nisin (4 μ g ml⁻¹) was used as a positive control, while vancomycin (4 μ g ml⁻¹), SDW and the highest concentration of DMSO used in the experiment were included as negative controls. Each assay was repeated three times and the mean and SD determined.

Leakage of 260-nm-absorbing material

The assay was carried out as previously described (Carson *et al.* 2002). The bacterial suspensions in PBS (pH 7.3) were exposed to different concentrations of treatments and were incubated anaerobically at 35°C. At time 0, 1, 2 and 6 h postincubation, 1 ml aliquots were removed from each treatment, diluted 1 in 10 in PBS (pH 7.3) and filtered through a 0.22- μ m pore size filter. The absorbance for the filtrates was measured in triplicate at 260 nm. Filtrates of the appropriate dilution of each treatment were prepared and used as blanks. Aliquots of 200 μ l in UV-transparent 96-well trays (Molecular Devices, Sunnyvale, CA) were used for both the loss of 260-nm-absorbing material and the bacteriolysis assays, and the OD was measured using an xMark™ microplate spectrophotometer.

Protein-leakage assay

Protein leakage was analysed using a Bradford protein assay (Tiwari *et al.* 2008). The bacterial suspensions were prepared in PBS (pH 7.3). Aliquots of 1 ml were removed from each treatment at 0, 1, 2 and 6 h postincubation anaerobically at 35°C and supernatants were collected after centrifugation of samples at 4032 g min⁻¹ for 10 min. For each sample, 100 μ l of supernatant was mixed with 100 μ l of Bradford working solution in a 96-well tray. The OD₅₉₅

was measured in triplicate after a 10-min incubation in the dark. Supernatants of the appropriate dilution of each treatment were prepared and used as blanks. Bovine serum albumin was used as the standard protein with a known concentration to construct a standard curve (Fig. S1). The absorbance values were used to determine the concentration of leaked protein via the linear regression line of the standard curve.

Propidium iodide uptake

The assay was performed as described by Wu *et al.* (2013) with slight modifications. The stationary-phase cells in BHIB were exposed to treatments at various concentrations and incubated anaerobically at 35°C. Samples (1 ml) were removed after 1, 2 and 6 h. Each sample was centrifuged at 4032 g min⁻¹ for 10 min and the cell pellet was resuspended in 1 ml working solution containing 5 µmol l⁻¹ propidium iodide. Samples were incubated anaerobically at 35°C for 10 min, centrifuged and washed with PBS to remove the excess dye. Propidium iodide fluorescence in cells was measured in a FluoStar Optima Plate Reader (BMG Labtech) at an excitation of 544 nm and emission of 620 nm.

Protein synthesis inhibition assay

An *in vitro* transcription/translation experiment was carried out using Promega's *Escherichia coli* S30 extract system for circular DNA linked to the Steady-Glo[®] Luciferase assay system according to the manufacturer's instruction. The assay was performed using fresh garlic extract, *Leptospermum* honey (MGO 514⁺) and allicin, all of which showed bacteriostatic activity against *C. difficile*. Streptomycin sulphate and tetracycline were included as positive controls (inhibiting protein synthesis). Briefly, enough master mix for the transcription/translation reactions was prepared by combining the reaction components included in the kit. Each reaction contained 49 µl of the master mix and 1 µl of the treatment and was incubated at 37°C for 60 min. After incubation, the reaction was stopped by placing the tubes on the ice for 5 min. To detect the luciferase protein, 95 µl of SDW was added to 5 µl of each translation reaction in a white opaque 96-well microplate, followed by the addition of 100 µl of Steady-Glo[™] reagent. Luminescence was measured in a POLARstar Optima plate reader (BMG Labtech). Background luminescence (SDW only) was subtracted from the experimental luminescence. The relative inhibition of protein synthesis was expressed as a percentage of net luminescence in the presence of treatment over the net luminescence in the absence of treatment. Luminescence was measured in terms of relative

light units (RLU). The assay was repeated three times and the mean and SD determined.

Detection of antimicrobial cross-resistance

The activity of the nine products/components showing either bactericidal or bacteriostatic effects was determined against a panel of *C. difficile* strains previously characterized for AMR phenotype and associated genotype. The panel comprised strains determined to be (i) fluoroquinolone-resistant (FQR), mediated by DNA gyrase mutations; (ii) tetracycline-resistant (tetR), mediated by conjugative transposons Tn5397 and Tn6190 carrying *tetM*; and (iii) resistant to erythromycin and clindamycin (macrolide-lincosamide-streptogramin-B (MLS_B) phenotype, mediated by conjugative transposon Tn6194 carrying *ermB* (Knight *et al.* 2016). MICs for each product/component were determined on three separate occasions and a modal value selected. The metronidazole- and vancomycin-tolerant isolates were generated using a serial passaging method (Mascio *et al.* 2014).

Statistical analysis

Statistical differences between three or more sets of data were analysed with GraphPad Prism ver. 7 software using one-way analysis of variance and nonparametric techniques, followed by Tukey's multiple comparison post-test if the *P* value was significant. The Student's two-tailed *t*-test assuming unequal variance was used to determine whether there was a significant difference between two sets of data. *P* values of <0.05 were considered significant.

Results

The MICs and MBCs of natural products and antimicrobial controls against both stationary- and log-phase *C. difficile* have previously been published (Roshan *et al.* 2017, 2018a,b).

Time-kill assay

Five products/components previously shown to have bactericidal activity against stationary-phase *C. difficile* (Roshan *et al.* 2017) were tested. These were cinnamon root powder, peppermint oil, *trans*-cinnamaldehyde, menthol and zingerone. Exposure to these products led to substantial reductions in viability of both *C. difficile* strains after 24 h (Tables 1 and S2). Peppermint oil at ≥1× MIC resulted in a log₁₀ 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

Table 1 Mean log10 CFU per ml reductions for *Clostridium difficile* NCTC 13366 following exposure to products

Treatment	Concentration	<i>Clostridium difficile</i> NCTC 13366											
		1 h			2 h			4 h			6 h		
		EXP	STA	EXP	STA	EXP	STA	EXP	STA	EXP	STA	EXP	STA
Cinnamon root powder	1 × MIC	0.6 ± 0.3	1.5 ± 0.4	1.2 ± 0.2	1.6 ± 0.4	1.2 ± 0.3	2.1 ± 0.2	1.3 ± 0.2	2.4 ± 0.3	1.6 ± 0.2	2.6 ± 0.2	1.6 ± 0.3	2.6 ± 0.2
	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 ± 0.2	3.6 ± 0.4	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	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	3.8 ± 0.4	NA
Peppermint oil	1 × MIC	1.9 ± 0.1	1.4 ± 0.4	2.2 ± 0.2	1.7 ± 0.3	2.6 ± 0.3	2.2 ± 0.4	3.0 ± 0.1	2.4 ± 0.3	3.4 ± 0.1	3.4 ± 0.5	3.4 ± 0.1	3.4 ± 0.5
	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	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	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	3.9 ± 0.2	NA
<i>trans</i> -Cinnamaldehyde	1 × MIC	2.4 ± 0.8	0.7 ± 0.4	2.3 ± 0.9	1.8 ± 0.3	2.9 ± 0.5	2.0 ± 0.4	3.0 ± 0.5	2.6 ± 0.2	3.0 ± 0.6	2.9 ± 0.2	3.0 ± 0.6	2.9 ± 0.2
	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	4.2 ± 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	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	4.2 ± 0.2	3.3 ± 0.3
Menthol	1 × MIC	0.5 ± 0.3	1.3 ± 0.8	1.2 ± 0.3	2.0 ± 0.2	1.5 ± 0.4	2.4 ± 0.2	2.4 ± 1.0	2.4 ± 0.2	2.9 ± 0.8	3.5 ± 0.4	2.9 ± 0.8	3.5 ± 0.4
	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	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	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 ± 0.1	3.2 ± 0.5	4.0 ± 0.1	3.2 ± 0.4	4.1 ± 0.2	3.9 ± 0.1	4.1 ± 0.2	3.9 ± 0.1
Zingerone	1 × MIC	0.9 ± 0.2	0.6 ± 0.3	1.6 ± 0.4	0.9 ± 0.1	2.1 ± 0.8	1.1 ± 0.6	1.8 ± 0.5	1.3 ± 0.4	-0.1 ± 0.7	-0.1 ± 0.2	-0.1 ± 0.7	-0.1 ± 0.2
	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	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	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	4.2 ± 0.2	3.3 ± 0.5
SDW	-	-0.1 ± 0.1	-0.03 ± 0.1	-0.2 ± 0.1	-0.2 ± 0.3	-0.4 ± 0.1	-0.3 ± 0.3	-0.8 ± 0.3	-0.6 ± 0.3	-1.5 ± 0.2	-1.6 ± 0.5	-1.5 ± 0.2	-1.6 ± 0.5
DMSO	2.5% v/v	-0.3 ± 0.3	-0.1 ± 0.2	-0.43 ± 0.4	-0.4 ± 0.3	-0.7 ± 0.4	-0.7 ± 0.4	-0.9 ± 0.5	-0.9 ± 0.3	-1.9 ± 0.3	-1.7 ± 0.1	-1.9 ± 0.3	-1.7 ± 0.1

EXP, exponential; STA, stationary; MIC, minimum inhibitory concentration; NA, not applicable; SDW, sterile distilled water; DMSO, dimethyl sulfoxide. MICs were as previously stated in Roshan et al. (2018a,b).

with *trans*-cinnamaldehyde at nearly all concentrations. The only exception was *trans*-cinnamaldehyde at $1\times$ MIC against stationary-phase *C. difficile* NCTC 13366, where the $\log_{10}$ reduction was 2.9 after 24 h. For zingerone and menthol, a $\log_{10}$ reduction of ≥ 3 was obtained at $\geq 2\times$ MIC and $\geq 4\times$ MIC, respectively, against both log- and stationary-phase *C. difficile* after 24 h of exposure. Cinnamon root powder at $\geq 4\times$ MIC (≥ 75 mg ml⁻¹) resulted in a marked reduction in bacterial counts (a $\log_{10}$ reduction of ≥ 3.2) against both log-phase *C. difficile* strains and a $\log_{10}$ reduction of ≥ 3.5 at $\geq 2\times$ MIC (≥ 75 mg ml⁻¹) against stationary-phase *C. difficile* after 24 h.

ATP-leakage assay

Effects of the five natural products/components on ATP leakage from logarithmic-phase cultures of *C. difficile* NCTC 13366 are shown in Figs 1 and S2. While nisin ($4\text{ }\mu\text{g ml}^{-1}$) caused significant leakage of ATP compared to the untreated controls (SDW and DMSO), as observed by both the intracellular and extracellular level of ATP ($P < 0.001$), vancomycin ($8\text{ }\mu\text{g ml}^{-1}$) did not cause any ATP leakage after 2 h. Exposure to all five natural products/components at most concentrations resulted in a significant decrease in intracellular ATP only after 1 h ($P \leq 0.013$). The only exception was cinnamon root powder and zingerone at the lower concentration of $1\times$ MIC, which did not show any significant extracellular ATP increase. Exposure to menthol and peppermint oil at $4\times$ MIC and $8\times$ MIC resulted in significant leakage of extracellular ATP after 1 h of exposure ($P < 0.04$). Most treatments at concentrations of $\leq 2\times$ MIC did not induce any significant increase in extracellular ATP up to 2 h ($P \geq 0.06$).

Bacteriolysis

None of the treatments induced lysis of bacterial cells (Table S3). However, the positive control triton X-100

(10% v/v) reduced the OD_{620 nm} to 64.9% after 6 h for both strains, whereas values for the untreated control (SDW) ranged from 85.0 to 85.6%.

Permeability assays

The results from the three different cell permeability assays are shown in Table 2 and S4. The five natural products/components were tested against stationary-phase cultures of *C. difficile* ATCC 700057 and NCTC 13366.

Loss of 260-nm-absorbing material

No significant increase in OD₂₆₀ was observed for any of the treatments after 1 h of exposure, compared to the untreated controls (SDW and DMSO). The exception was the positive control, nisin ($4\text{ }\mu\text{g ml}^{-1}$) against *C. difficile* strain NCTC 13366 ($P < 0.03$). A longer incubation time (6 h) resulted in a significant increase in OD₂₆₀ when *C. difficile* strains ATCC 700057 and NCTC 13366 were exposed to $\geq 2\times$ MIC of treatments ($P < 0.05$). However, no significant leakage of 260-nm-absorbing material was observed with cinnamon root powder ($P > 0.05$).

Protein-leakage assay

Treatment with peppermint oil, *trans*-cinnamaldehyde, menthol and zingerone resulted in a significant increase in OD₅₉₅ indicating protein leakage after 1 h of exposure compared to the untreated controls (SDW and DMSO) ($P \leq 0.04$). Cinnamon root powder after 6 h of incubation at $2\times$ MIC (150 mg ml^{-1}) resulted in a significant increase in extracellular protein ($P < 0.01$).

Propidium iodide uptake assay

As expected, nisin-treated *C. difficile* cells were stained with propidium iodide after 1 h of exposure, which signified a decrease in membrane integrity of *C. difficile* cells. The relative fluorescence unit (RFU) of propidium iodide

Figure 1 Effect (mean $\pm$ SD) of five bactericidal products and comparators on the leakage of ATP from *Clostridium difficile* NCTC 13366 (■ nisin; ▲ vancomycin; ▼ cinnamon; ◆ peppermint oil; ○ *trans*-Cinnamaldehyde; □ menthol; △ zingerone).

Table 2 Effect of products on membrane integrity of *Clostridium difficile* NCTC 13366, measured by increase in extracellular 260-nm-absorbing materials, protein leakage and propidium iodide uptake assays

Treatment	Extracellular 260-nm-absorbing material (OD ₂₆₀)			Protein-leakage assay (protein leakage mg ml ⁻¹)			Propidium iodide uptake (RFUs)		
	1 h	2 h	6 h	1 h	2 h	6 h	1 h	2 h	6 h
<i>Cinnamon root powder</i>									
1 × MIC (75 mg ml ⁻¹)	0.03 ± 0.02	0.06 ± 0.03	0.07 ± 0.02	7.0 ± 2.3	3.0 ± 1.8	24.8 ± 5.7	324 ± 53	376 ± 136	602 ± 128
2 × MIC (150 mg ml ⁻¹)	0.03 ± 0.02	0.07 ± 0.02	0.11 ± 0.01	4.7 ± 5.0	24.4 ± 10.6	83.1 ± 10.6	446 ± 64	616 ± 168	1061 ± 228
<i>Peppermint oil</i>									
1 × MIC (8% v/v)	0.01 ± 0.00	0.04 ± 0.02	0.11 ± 0.01	19.2 ± 6.8	23.6 ± 5.2	74.3 ± 3.9	241 ± 60	444 ± 108	1206 ± 186
2 × MIC (16% v/v)	0.02 ± 0.01	0.05 ± 0.01	0.14 ± 0.02	29.6 ± 9.1	62.9 ± 2.7	90.7 ± 11.2	431 ± 156	548 ± 111	1387 ± 294
4 × MIC (32% v/v)	0.03 ± 0.01	0.08 ± 0.02	0.24 ± 0.08	32.8 ± 11.0	74.1 ± 2.6	99.5 ± 8.7	256 ± 14	399 ± 142	1061 ± 228
<i>trans-Cinnamaldehyde</i>									
1 × MIC (0.02% v/v)	0.01 ± 0.00	0.04 ± 0.02	0.07 ± 0.01	0.0 ± 0.0	0.2 ± 0.3	2.2 ± 3.8	548 ± 156	854 ± 181	523 ± 11
2 × MIC (0.04% v/v)	0.02 ± 0.02	0.06 ± 0.02	0.12 ± 0.02	0.0 ± 0.0	3.9 ± 3.9	6.9 ± 5.9	724 ± 43	1831 ± 69	745 ± 15
4 × MIC (0.08% v/v)	0.01 ± 0.01	0.03 ± 0.01	0.15 ± 0.01	0.0 ± 0.0	9.1 ± 7.4	18.2 ± 8.1	985 ± 22	1186 ± 37	618 ± 8
8 × MIC (0.16% v/v)	0.02 ± 0.01	0.05 ± 0.00	0.17 ± 0.07	27.2 ± 14.9	31.0 ± 21.3	45.9 ± 12.7	1115 ± 46	1446 ± 70	1444 ± 49
<i>Menthol</i>									
1 × MIC (9.4 mg ml ⁻¹)	0.01 ± 0.00	0.02 ± 0.01	0.05 ± 0.00	0.0 ± 0.0	36.0 ± 6.9	42.2 ± 17.6	308 ± 12	315 ± 99	223 ± 21
2 × MIC (18.8 mg ml ⁻¹)	0.02 ± 0.01	0.04 ± 0.01	0.11 ± 0.01	24.6 ± 14.8	37.2 ± 12.6	51.1 ± 13.3	287 ± 21	287 ± 88	487 ± 99
4 × MIC (37.5 mg ml ⁻¹)	0.02 ± 0.01	0.04 ± 0.02	0.12 ± 0.02	37.7 ± 4.8	51.5 ± 16.7	62.3 ± 22.6	337 ± 9	463 ± 101	513 ± 88
8 × MIC (75 mg ml ⁻¹)	0.04 ± 0.03	0.06 ± 0.02	0.14 ± 0.03	24.2 ± 8.9	48.8 ± 20.1	76.6 ± 13.5	461 ± 84	499 ± 88	987 ± 44
<i>Zingerone</i>									
1 × MIC (9.4 mg ml ⁻¹)	0.02 ± 0.00	0.05 ± 0.02	0.08 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	698 ± 73	595 ± 20	990 ± 99
2 × MIC (18.8 mg ml ⁻¹)	0.02 ± 0.01	0.03 ± 0.01	0.12 ± 0.01	11.5 ± 5.4	5.2 ± 3.3	3.1 ± 3.3	739 ± 67	574 ± 19	1247 ± 29
4 × MIC (37.5 mg ml ⁻¹)	0.04 ± 0.02	0.06 ± 0.01	0.16 ± 0.07	5.7 ± 3.3	20.3 ± 9.6	35.2 ± 15.3	1263 ± 425	919 ± 21	1301 ± 12
8 × MIC (75 mg ml ⁻¹)	0.03 ± 0.01	0.06 ± 0.02	0.16 ± 0.03	38.9 ± 13.9	50.7 ± 13.4	41.7 ± 15.1	1101 ± 418	1601 ± 122	1214 ± 57
Nisin (4 µg ml ⁻¹)	0.06 ± 0.02	0.10 ± 0.00	0.16 ± 0.04	6.7 ± 4.1	29.8 ± 5.6	53.7 ± 11.4	1448 ± 59	1254 ± 35	3611 ± 284
Vancomycin (8 µg ml ⁻¹)	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.01	4.1 ± 2.4	16.7 ± 4.7	18.5 ± 7.2	181 ± 16	175 ± 54	218 ± 88
SDW	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.02	0.0 ± 0.0	0.1 ± 0.1	1.6 ± 2.9	147 ± 26	185 ± 45	206 ± 65
DMSO (2.5% v/v)	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 2.1	134 ± 37	185 ± 45	166 ± 53

SDW, sterile distilled water; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; RFUs, relative fluorescence units. Values in bold differ significantly from controls (SDW and DMSO), *P* < 0.05.

was 1448 ± 59 for nisin-treated *C. difficile* NCTC 13366, compared with a value of 147 ± 26 for untreated control cells (SDW) ($P < 0.001$). Similar results were observed with nisin-treated *C. difficile* ATCC 700057 cells (RFU = 1572 ± 456 for nisin vs 111 ± 23 for untreated control). Treatment with zingerone and *trans*-cinnamaldehyde at $\geq 1 \times$ MIC resulted in significant propidium iodide uptake by *C. difficile* cells after 1 h. Treatment with all natural products/components at most tested concentrations resulted in a significant increase in propidium iodide fluorescence after 6 h of exposure. Vancomycin was used as a negative control in all three cell permeability assays and no evidence of cell leakage or increased permeability was observed for either *C. difficile* strain.

Inhibition of protein synthesis

The three products with bacteriostatic activity against *C. difficile* (allicin, fresh garlic extract and *Leptospermum* honey (MGO 514⁺) were tested in this experiment at various concentrations (Table 3). Streptomycin sulphate and tetracycline were used as positive controls and both inhibited protein synthesis/prokaryotic translation. Of the natural products, *Leptospermum* honey (MGO 514⁺) was the only treatment that showed a reduction in RLU ratio compared to untreated controls (SDW and DMSO) with values ranging from 42.1 to 68.9%.

Detection of antimicrobial cross-resistance

In this experiment, the MICs of natural products/components were determined against an additional panel of *C. difficile* strains showing combinations of FQR, tetR and MLS_B resistance or susceptibility (Table S1). The *in vitro* activity of the nine natural products/components is shown in Table 4. Overall, no more than twofold difference in MIC was observed between the resistant (FQR⁺, tetR⁺ and MLS_B⁺) and susceptible (FQR⁻, tetR⁻ and MLS_B⁻) strains, indicating the absence of cross-resistance and alternative mechanisms of action for the tested treatments. Moreover, no difference in MIC was shown between the parent strains and those that had been serially passaged against metronidazole or vancomycin (data not shown).

Discussion

The current antimicrobial therapy for CDI has limitations either due to failure to prevent recurrence or high cost (Huang *et al.* 2009). Therefore, there is a need for better treatment options to effectively manage infection. In particular, determining target sites of antimicrobials within *C. difficile* cells is of great importance for discovering new

Table 3 Effect of products on inhibition of protein synthesis using the *Escherichia coli* S30 extract system

Test products	Test concentration	RLU ratio % (mean $\pm$ SD)
<i>Natural products</i>		
Fresh garlic extract	50% v/v	79.9 $\pm$ 14.5
Fresh garlic extract	25% v/v	89.6 $\pm$ 6.4
Fresh garlic extract	12.5% v/v	101.5 $\pm$ 5.6
Allicin	75 mg ml ⁻¹	102.2 $\pm$ 5.4
Allicin	37.5 mg ml ⁻¹	101.5 $\pm$ 3.1
Allicin	18.8 mg ml ⁻¹	103.7 $\pm$ 2.2
<i>Leptospermum</i> honey (MGO 514 ⁺)	32% w/v	42.1 $\pm$ 11.8
<i>Leptospermum</i> honey (MGO 514 ⁺)	16% w/v	61.2 $\pm$ 7.3
<i>Leptospermum</i> honey (MGO 514 ⁺)	8% w/v	68.9 $\pm$ 6.6
<i>Controls</i>		
Streptomycin	1 mg ml ⁻¹	0.4 $\pm$ 0.4
Streptomycin	0.1 mg ml ⁻¹	0.6 $\pm$ 0.4
Streptomycin	0.01 mg ml ⁻¹	6.4 $\pm$ 3.5
Tetracycline	1 mg ml ⁻¹	1.6 $\pm$ 1.4
Tetracycline	0.1 mg ml ⁻¹	49.5 $\pm$ 4.1
Tetracycline	0.01 mg ml ⁻¹	91.6 $\pm$ 6.4
SDW		100 $\pm$ 0.0
DMSO	2.5% v/v	102.4 $\pm$ 4.1

RLU, relative light unit; SD, standard deviation; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

Values in bold differ significantly from controls (SDW and DMSO), $P < 0.05$.

anti-*C. difficile* therapeutic agents (Wu *et al.* 2013). In this follow-up study, the mode of action of nine natural products/components with bactericidal or bacteriostatic activity against *C. difficile* was investigated. Time-kill assays were performed with the five bactericidal products/components to further investigate their mode of killing against *C. difficile*. Cinnamon and peppermint oil in addition to three pure compounds, *trans*-cinnamaldehyde, menthol and zingerone, showed a $>3 \log_{10}$ reduction in *C. difficile* viable counts following 24 h of exposure, further demonstrating their bactericidal activity. In general, cinnamon, menthol and zingerone showed a concentration-dependent mode of killing, while killing trends for peppermint oil and *trans*-cinnamaldehyde were less obvious. Investigation of the levels of intracellular and extracellular ATP after exposure of log-phase *C. difficile* to treatments showed depletion of cellular ATP which further confirmed their bactericidal action against *C. difficile*. To our knowledge, no published studies have previously shown the mechanism of action, including the time-kill kinetics, for natural products/components against *C. difficile*. However, the mode of action and the bactericidal properties of surotomycin as a membrane-active compound have previously been assessed. Surotomycin showed concentration-dependent killing of both logarithmic- and stationary-phase cultures of *C. difficile*

Table 4 MIC values for products against antimicrobial-resistant and susceptible *Clostridium difficile*

		MIC (mg l ⁻¹)					Treatment							
solate ID	Phenotype	CLl	ERY	MXF	TET	Fresh garlic extract (% v/v)	Garlic clove powder (mg ml ⁻¹)	Allicin (mg ml ⁻¹)	Leptospermum honey (MGO 514+) (% w/v)	Peppermint oil (% v/v)	Cinnamon root powder (mg ml ⁻¹)	Menthol (mg ml ⁻¹)	Zingerone (mg ml ⁻¹)	trans-cinnamaldehyde (%v/v)
ESP123	MLS _B /tetR	>32	>256	1	32	0.4	9.4	9.4	8	4	75	4.7	9.4	0.02
SQ383		0.12	1	2	0.06	0.8	9.4	9.4	8	8	75	9.4	9.4	0.03
SQ357	MLS _B	8	>256	2	0.06	0.8	9.4	9.4	16	8	75	9.4	9.4	0.01
SQ463		2	1	2	0.06	0.8	9.4	9.4	16	8	75	9.4	9.4	0.02
SAP019	MLS _B /tetR	16	>256	1	32	0.8	4.7	9.4	8	8	75	9.4	9.4	0.03
SQ317		0.12	0.06	0.5	0.06	0.8	9.4	9.4	16	8	75	9.4	9.4	0.03
ES0474	FOR/tetR	1	1	16	16	0.8	9.4	9.4	16	8	75	9.4	9.4	0.03
ES1046		0.12	0.5	1	0.06	0.8	9.4	9.4	16	8	75	9.4	9.4	0.03

MGO, methylglyoxal; MLS_B, macrolide-lincosamide-streptogramin-B; tetR, tetracycline-resistant; FOR, fluoroquinolone-resistant. Italic and bold values indicate susceptible and resistant MIC values, respectively.

MGO, methylglyoxal; MLS_B, macrolide-lincosamide-streptogramin-B; tetR, tetracycline-resistant; FQR, fluoroquinolone-resistant. Italic and bold values indicate susceptible and resistant MIC values, respectively.

(Alam *et al.* 2015). Another study investigated the bactericidal activities of various membrane-active agents such as nigericin, valinomycin, clofazimine and daptomycin (Wu *et al.* 2013). Most of the agents (and to a lesser extent daptomycin) showed a concentration-dependent killing of both log- and stationary-phase *C. difficile* with simultaneous reductions in cellular ATP (Wu *et al.* 2013). In that study, nisin, metronidazole and vancomycin were used as controls and their effects on the ATP synthesis were consistent with our findings, whereby nisin and metronidazole reduced intracellular ATP ($P \leq 0.01$), while vancomycin showed no significant effect on ATP leakage ($P \leq 0.119$). In another study, the effect of metronidazole, vancomycin and nisin on ATP synthesis was investigated and effects were again consistent with our findings, whereby nisin and metronidazole reduced intracellular ATP ($P \leq 0.01$), while vancomycin showed no significant effect on ATP leakage ($P \leq 0.119$). A study by Corbett *et al.* (2015) investigated the killing kinetics for SMT19969, a novel antimicrobial under development at the time of study, along with the comparator antimicrobials fidaxomicin and vancomycin. SMT19969 showed a $>3 \log_{10}$ reduction in viable counts after 24 h of exposure. However, killing of *C. difficile* by SMT19969 was independent of drug concentration, while fidaxomicin showed a time-dependent mode of killing. As expected, vancomycin was bacteriostatic at all concentrations (Corbett *et al.* 2015).

Several antimicrobial agents provoke whole cell lysis by causing cell wall damage, as reported for essential oils from thyme, oregano and rosewood against *Streptococcus pneumoniae* (Horne *et al.* 2001; Carson *et al.* 2002). However, in this current study, none of the products resulted in lysis of *C. difficile* cells. In contrast, results from the cell permeability assays showed that all five bactericidal products/components damaged the cell membrane, as shown in two or more of the cell permeability assays. Maintaining membrane integrity is critical for the survival of logarithmic- and stationary-phase cells of *C. difficile*, hence the clostridial membrane is considered to be an important target for treatment of CDI (Bush 2012; Wu *et al.* 2013). In general, agents disrupting membrane integrity also show rapid bactericidal activity (Wu *et al.* 2013). Nazzaro *et al.* (2013) stated that essential oils and their components have activity against a variety of targets, mainly the cytoplasmic membrane, which was also observed in this study. In particular, it has been suggested that *trans*-cinnamaldehyde at lethal concentrations damages the cell membrane (Nazzaro *et al.* 2013). Many membrane-targeting agents, including chlorhexidine and phenethyl alcohol (Hugo and Longworth 1964) and tea tree oil (Carson *et al.* 2002), induce the loss of 260-nm-absorbing material in a range of organisms. In this study,

loss of 260-nm-absorbing material was shown for all products/components except cinnamon root, which suggests the loss of nucleic acids via a damaged cytoplasmic membrane. However, a high concentration and long exposure time were required to show significant leakage of intracellular material. On the other hand, measuring the leakage of intracellular protein by the Bradford assay showed significant protein leakage for all five treatments after a shorter exposure time. Additionally, the propidium iodide uptake assay showed disruption of membrane integrity by all five bactericidal products, which is associated with loss of integrity in the cytoplasmic membrane of *C. difficile* cells (Wu *et al.* 2013). This difference is likely because the uptake of propidium iodide is a more sensitive assay than the leakage of 260 nm materials and is able to detect relatively minor changes in membrane integrity.

Since one of the frequent mechanisms of action for bacteriostatic compounds is the inhibition of protein synthesis (Zubay 1973; Arenz and Wilson 2016), this was investigated using an *E. coli* S30 extract system capable of *in vitro* transcription and translation. Previously, tetracycline and streptomycin were shown to inhibit prokaryotic translation (Arenz and Wilson 2016), and in this study both agents served as positive controls and both showed inhibition of protein synthesis with streptomycin having a greater effect. *Leptospermum* honey also showed a reduction in RFU ratio indicating a likely impact on protein synthesis. The multicomponent nature of honey means that several factors contribute to its overall activity (Aljarallah 2016; Rossiter *et al.* 2017). The effect of *Leptospermum* honeys on the suppression of protein synthesis has been also mentioned in previous microarray and proteomic studies of bacteria (Packer *et al.* 2012; Carter *et al.* 2016).

Over time, *C. difficile* has developed resistance to several different classes of antimicrobials; however, the rate varies widely depending on geographic regions and antimicrobial use policies (Spigaglia 2016). The emergence of AMR in *C. difficile* is mainly due to alteration of antimicrobial targets and the presence of resistant-associated genes in the bacterial chromosome (Spigaglia 2016). Coordinated efforts are required to manage the AMR crisis and implement new and alternative therapeutic options with less chance of acquiring resistance. In the current study, all nine natural products/components were tested against a panel of *C. difficile* strains showing combinations of FQR, tetR and MLS_B resistance or susceptibility. Notably, none of the natural products showed increased MICs against strains with FQR, tetR and MLS_B resistance. This suggests that the mode of action for these natural products/components may be different to the known modes of action of fluoroquinolones (targeting DNA gyrase), tetracyclines

(targeting riboprotein proteins) and macrolides/lincosamides (targeting 23s rRNA); however, further investigations are required to confirm this. With regard to bacteria developing resistance to natural products, studies indicate that this unlikely to occur (Yap *et al.* 2014). In a study by Blair *et al.* (2009) serial passaging of *Staphylococcus aureus* and *Pseudomonas aeruginosa* with active *Leptospermum* honey or the antimicrobials tetracycline and ciprofloxacin was conducted; however, no resistance to honey could be induced under the same conditions that rapidly induced resistance to antimicrobials (Blair *et al.* 2009). Therefore, compounds derived from plants may have great potential as antimicrobials against drug-resistant micro-organisms.

Many natural products, including those used in this study, have 'Generally Recognized as Safe' (GRAS) status with the US Food and Drug Administration. However, as side effects may be expected with some if consumed in large quantities (U.S. Food and Drug Administration: GRAS Substances), consumption must be moderated. It is necessary to identify risks associated with the use or ingestion of plant-derived compounds, so that any toxic compounds can be identified, and either discarded or modified to provide a safer alternative (World Health Organisation, 2004). Very few natural products, including those investigated in this study, have been rigorously studied in animal studies or human clinical trials for effectiveness in preventing or treating gastrointestinal infections. As such, the potential therapeutic benefits of natural products remain largely unknown.

Overall, this study provides a fundamental framework regarding the possible mechanism of action of naturally occurring products against *C. difficile*. Whilst the cell wall appeared to be unaffected, several products showed damage to the clostridial membrane and resulted in depletion of intracellular ATP in *C. difficile*. These factors are important targets for investigating anti-*C. difficile* drugs to reduce the burden of CDI. Also, as it is less likely to be cross-over mechanisms of resistance between standard antimicrobials and natural products, this further increases the potential for reducing the rate of AMR. Future work is still required to better understand the mechanisms involved and to assess the effect of these natural products *in vivo*.

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Conflict of Interest

No conflict of interest is declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Antibigrams for selected *C. difficile* isolates

Table S2 Mean log₁₀ CFU per ml reductions for *C. difficile* ATCC 700057 following of exposure to treatments

Table S3 Bacteriolysis results

Table S4 Effect of products on membrane integrity of *C. difficile* ATCC 700057, measured by increase in extracellular 260-nm-absorbing materials, protein leakage and propidium iodide uptake assays

Figure S1 Standard curve of BSA (mg ml⁻¹) measured at OD₅₉₅.

Figure S2 Effect (mean ± SD) of five bactericidal products and comparators in the leakage of ATP from the *C. difficile* cells, strain NCTC 13366.

Antibacterial activity and chemical characteristics of several Western Australian honeys compared to manuka honey and pasture honey

Niloufar Roshan¹ · Thomas Rippers² · Cornelia Locher² · Katherine A. Hammer¹

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Abstract The physicochemical parameters and antibacterial activity of 10 Western Australian (WA) and two comparator honeys were determined. Honeys showed a pH range of 4.0–4.7, colour range of 41.3–470.7 mAU, methylglyoxal levels ranging from 82.2 to 325.9 mg kg⁻¹ and hydrogen peroxide levels after 2 h of 22.7–295.5 µM. Antibacterial activity was assessed by the disc diffusion assay, phenol equivalence assay, determination of minimum inhibitory and bactericidal concentrations and a time-kill assay. Activity was shown for all honeys by one or more method, however, activity varied according to which assay was used. Minimum inhibitory concentrations for WA honeys against 10 organisms ranged from 4.0 to >32.0% (w/v). Removal of hydrogen peroxide activity by catalase resulted in decreased activity for several honeys. Overall, the data showed that honeys in addition to those derived from *Leptospermum* spp. have antimicrobial activity and should not be overlooked as potential sources of clinically useful honey.

Keywords Apitherapy · Methylglyoxal · *Eucalyptus* · *Banksia* · Jarrah · Marri

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✉ Katherine A. Hammer
katherine.hammer@uwa.edu.au

¹ School of Pathology and Laboratory Medicine (M504), The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia

² School of Medicine and Pharmacology (M315), The University of Western Australia, 35 Stirling Hwy, Crawley, WA 6009, Australia

Introduction

Honey is a natural substance produced by *Apis mellifera* honeybees using nectar collected from flowers or other parts of plants. It is not only a food, but is also a medicinal product, with historical medicinal use based largely on its antimicrobial activity (Cooper and Jenkins 2012). Several factors in honey contribute to the antibacterial activity including high osmolarity, low pH, the production of hydrogen peroxide and phytochemical factors. In recent years, methylglyoxal has been identified as an important phytochemical in *Leptospermum* honeys, and in particular manuka honey from *L. scoparium* (Adams et al. 2008). This is one of only a select few honeys that have been developed into medical products suitable for clinical use.

Australia is home to a diverse and unique natural flora. The southwest region of Western Australia, in particular, is an internationally recognised biodiversity hotspot (Rix et al. 2015) and has a large number of unique, endemic plants, several of which are important to the honey industry. Monofloral honeys important to the Western Australian honey industry include Jarrah honey from *Eucalyptus marginata* and Marri honey from *Corymbia calophylla* (Dawes and Dall 2014). These honeys may also be therapeutically valuable but there are little published scientific data describing their antimicrobial activity and supporting their medicinal use.

A major study by Irish et al. (2011) evaluated 477 Australian honeys, including 96 honeys from Western Australia. Their study identified the two WA honeys Jarrah and Marri as having high antibacterial activity, determined using the phenol equivalence assay. This assay, whilst widely used for honey testing, gives relatively limited information on the activity of each honey and does not allow any inferences to be made about what concentrations of honey

Table 1 Physicochemical characteristics of honeys

Honey	Floral source ^a	MGO ^b (mg/kg)	pH	Colour (mAU)	Hydrogen peroxide concentration (μmol)	
					2 h	24 h
Banksia	<i>Banksia</i> spp.	82.2 ± 4.5	4.6 ± 0.0	138.7 ± 7.5	22.7 ± 11.6	11.5 ± 16.2
Blackbutt	<i>Eucalyptus patens</i>	71.5 ± 11.9	4.5 ± 0.1	134.0 ± 7.0	71.3 ± 1.6	0.1 ± 0.0
Bottlebrush	<i>Callistemon</i> spp.	90.2 ± 8.7	4.6 ± 0.0	188.7 ± 5.5	60.1 ± 4.6	0.1 ± 0.0
Jarrah (2015)	<i>Eucalyptus marginata</i>	141.8 ± 11.5	4.7 ± 0.0	149.3 ± 7.3	295.5 ± 90.0	0.1 ± 0.0
Jarrah (2013)	<i>Eucalyptus marginata</i>	145.5 ± 11.3	4.6 ± 0.0	132.7 ± 5.6	72.6 ± 21.3	0.1 ± 0.0
Marri	<i>Corymbia calophylla</i>	95.0 ± 3.2	4.4 ± 0.0	116.0 ± 2.7	69.7 ± 19.6	0.1 ± 0.0
Moort	<i>Eucalyptus platypus</i>	90.1 ± 7.0	4.5 ± 0.0	41.3 ± 3.1	260.1 ± 72.9	0.1 ± 0.0
Spring	<i>Banksia sessilis</i> , <i>Callistemon</i> spp.	137.0 ± 2.9	4.7 ± 0.0	162.3 ± 2.6	242.6 ± 91.8	0.1 ± 0.0
WA manuka	<i>Leptospermum subtenue</i>	325.9 ± 0.6*	4.5 ± 0.0	206.7 ± 8.1	89.3 ± 0.0	0.1 ± 0.0
Wandoo	<i>Eucalyptus wandoo</i>	93.2 ± 7.0	4.5 ± 0.0	54.7 ± 2.3	243.0 ± 4.0	0.1 ± 0.0
Activon	<i>Leptospermum</i> spp.	281.0 ± 3.2	4.0 ± 0.0*	470.7 ± 2.9*	50.5 ± 9.0	0.1 ± 0.0
Capilano	no specific floral source	87.3 ± 5.0	4.3 ± 0.1	60.3 ± 6.0	34.7 ± 21.9	0.1 ± 0.0

* Differs significantly from the remaining honeys by one-way ANOVA ($p < 0.05$)

^a Most honeys were identified on the label by a common plant name only. The most likely genus and/or species was then determined from the Florabase database [<https://florabase.dpaw.wa.gov.au/>], via the internet or from the supplier

^b MGO methylglyoxal

may be therapeutically useful or how long is required for honey to have a bactericidal effect. Other than this study, there are very few published studies describing the antimicrobial activity of Western Australian honeys. The aim of the current study was therefore to explore in more depth the antimicrobial activity of a number of Western Australian honeys. This was achieved by examining activity using a range of different antibacterial susceptibility testing assays, and then correlating antibacterial activity with different physicochemical parameters to determine whether there are any significant relationships. Antibacterial activity was investigated using the agar well diffusion assay, phenol equivalence assay (Allen et al. 1991; Irish et al. 2011) broth microdilution assay and time-kill assay (Boorn et al. 2010; Clinical and Laboratory Standards Institute 2015), and results compared to those obtained for both the commercially available Activon (medical-grade manuka) honey and a Western Australian pasture honey with no specific floral source.

Materials and methods

Honey samples

A total of ten Western Australian honeys from a range of floral sources were obtained in March 2015 (Table 1). The two Jarrah honeys included one harvested during the 2015 flowering period (Dec 2014 to Jan 2015) and one from the previous flowering period in 2013. Floral source

was determined by beekeepers according to the local flowering patterns at the time of honey harvest. Where only a common plant name was provided, the most likely genus or species was determined from the Florabase database (<https://florabase.dpaw.wa.gov.au/>). In addition, Activon medical-grade manuka honey (Advancis Medical, Nottingham, UK) and Capilano (pasture) honey (Capilano Honey Pty Ltd, Bayswater, Western Australia) were purchased for use as comparators. Honeys were stored at room temperature, protected from light in plastic or glass jars. Unless stated otherwise, honeys were prepared for assays by dissolving weight for volume (w/v) in sterile distilled water (SDW). For example, a 50% (w/v) solution was prepared by adding 5 ml of sterile distilled water to 5 g of honey. To aid mixing, solutions were incubated at 37 °C for 15 min and then thoroughly mixed to ensure homogeneity. Solutions were used within 1 h of preparation. Honeys were also screened by culturing on Blood agar to determine whether they contained endemic microbes that could interfere with subsequent assays.

Physicochemical properties

The methylglyoxal content of honeys was quantified using high-performance liquid chromatography as described by Windsor et al. (2012). The pH of each honey was determined as described by Bogdanov et al. (1997). Briefly, 7.5 ml of carbon dioxide-free water was added to 1 g of each honey, which was incubated at 37 °C for 15 min to aid mixing. A pH meter was used to measure the pH value of

each honey solution. The assay was repeated on two different occasions, and the mean pH value was reported to two decimal places for each honey.

The colour intensity in milli-absorbance units (mAU) was quantified as described previously (Bogdanov et al. 1997; Beretta et al. 2005). A 50% (w/v) solution of each honey was prepared in SDW and then passed through a 0.2- μ m pore-sized filter to eliminate large particles. The absorbance of each solution was determined at 450 and 720 nm using a Bio-Rad xMark spectrophotometer. The difference in absorbance between wavelengths was expressed as milli-absorbance units (mAU) by multiplying the difference by 1000. The assay was repeated three times, and the mean and standard deviation (SD) was calculated for each honey.

Hydrogen peroxide levels were determined using a previously described colorimetric assay (Kwakman et al. 2011). Briefly, each honey was prepared as a 30% (w/v) solution in SDW and incubated at room temperature (23 °C). Samples were then removed after 2 and 24 h and were incubated at room temperature with a reagent consisting of o-dianisidine, horseradish peroxidase type IV and phosphate buffer. The reaction was stopped after 5 min using 6 molar sulphuric acid, and the absorbance was measured at 450 nm.

Antimicrobial activity assays

Inocula were prepared by culturing isolates on Blood agar for 18–24 h at 35 °C unless stated otherwise. Growth was suspended in 0.85% saline, and the turbidity was adjusted to 0.5 McFarland ($\sim 10^8$ cfu/ml).

Honeys were screened for activity against *S. aureus* ATCC 25923 using the agar well diffusion assay. Briefly, Mueller–Hinton agar plates were swab inoculated with a bacterial suspension containing approx. 10^8 cfu/ml, wells of 8 mm diameter were cut into the agar, and 100 μ l of each unfiltered 50% honey solution was added to each well. The effect of filtration on activity was also assessed using two representative honeys, Blackbutt and Jarrah 2015. These two honeys were selected on the basis of initial results showing that they demonstrated large zones of growth inhibition. To evaluate the effect of filtration, a portion of each 50% honey solution was passed through a 0.2 μ m pore filter (Acrodisc® Pall Corporation, USA) and the remaining portion was tested without filtration. Agar plates were incubated at 35 °C for 18–24 h after which the zone of growth inhibition was measured.

The phenol equivalence assay was conducted as previously described with solutions of 25% (v/w) honey (Allen et al. 1991; Irish et al. 2011) with the modification that 90-mm round petri dishes were used instead of 23-cm

square petri dishes. The assay was scaled proportionally, with each petri dish containing a 16 ml volume of nutrient agar seeded with the adjusted overnight culture of *S. aureus* ATCC 25923, resulting in an agar depth equivalent to that in the original assay. Phenol equivalence determined in the absence of catalase enzyme was termed total activity and that observed in the presence of catalase enzyme was termed non-peroxide activity.

Minimum inhibitory concentrations (MICs) of honey were determined by the broth microdilution assay according to CLSI guideline M07-A10 (Clinical and Laboratory Standards Institute 2015). Briefly, filtered honey solutions (64%) were serially diluted to result in a range after inoculation of 32–1%. Final inocula concentrations were approximately 5×10^5 cfu/ml, and after incubation of the assay for 18–24 h at 35 °C, MICs were determined visually as the lowest concentration of honey preventing growth. The minimum bactericidal concentration (MBC) was determined by spot-inoculating 10 μ l volumes from non-turbid wells onto Mueller–Hinton agar and was the lowest concentration showing a 99.9% reduction in the inoculum. The effect of the removal of hydrogen peroxide on activity against *S. aureus* ATCC 25923 was also investigated by treating honey solutions with catalase. For this, 1 ml of each 64% honey solution was mixed with 50 μ l of catalase solution (1000 U/ml) and incubated at room temperature for 2 h before use. A 50 μ l aliquot of SDW was added to 1 ml of the remaining honey solution to match the dilution factor. Catalase solution alone did not show antimicrobial activity. All MICs/MBCs were determined a minimum of three times, and the mode was selected as the final value. The geometric mean of the MIC and MBC was calculated. For this, off-scale MICs (>32%) were converted to the next higher concentration (64%) to enable analyses.

For the time-kill assay, inocula of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were prepared by culturing organisms overnight in 10 ml TSB at 37 °C with shaking, then diluting to approx. 5×10^8 cfu/ml in 0.85% saline. Each honey was prepared at 50% in 0.85% saline and filter sterilised. Honey solutions were inoculated with test organisms resulting in a final honey concentration of 40% (w/v) and a final inoculum concentration of approx. 2.5×10^6 cfu/ml. A negative control of 0.85% saline inoculated as above was included. Inoculated honey solutions and controls were incubated at 37 °C with shaking. Samples were removed at 0, 1, 2 and 24 h, and viable counts were performed by serially diluting samples tenfold in 0.85% saline and spot-inoculating 10 μ l volumes in duplicate from each dilution onto nutrient agar. Agar plates were incubated at 37 °C for 18–24 h, and the number of surviving organisms was determined. Inocula spots with two or fewer colonies were disregarded. The limit of detection was 2×10^3 cfu/ml.

Statistical analysis

All assays were repeated on three separate occasions unless stated otherwise, and the mean and standard deviation were calculated. Sets of data with multiple missing values, due to either a lack of antibacterial activity or lack of microbial growth, were not statistically analysed. Data for each physicochemical characteristic were analysed by nonparametric one-way ANOVA. MICs and MBCs were converted to log₂ values and then analysed by one-way ANOVA (nonparametric) followed by Tukey's multiple comparison post-test. Power calculations (12 samples, $\alpha = 0.05$, $\beta = 0.5$) showed power values of 0.80 for the physicochemical analysis and 0.65 for the MIC and MBC data. MICs obtained for each honey in the presence and absence of catalase enzyme were analysed by the Student's two-tailed *t* test, assuming unequal variance. For all statistical analyses, *p* values of <0.05 were considered significant.

Results and discussion

This study examined the physicochemical properties and bioactivity of a number of Western Australian honeys. The pH of the honeys ranged from 4.03 to 4.69 (Table 1), and the pH of Activon honey was significantly lower than the remaining honeys ($p < 0.001$). Colour intensity ranged from 41 mAU for pale yellow Moort to 471 mAU for the dark brown Activon honey, which differed significantly from the remaining honeys ($p < 0.001$). Methylglyoxal content ranged from 71.5–325.9 mg/kg and was highest

in WA manuka honey, which differed significantly from the remaining honeys ($p < 0.001$). Activon had the second highest MGO content (281.0 mg/kg), followed by Spring, Jarrah 2015 and Jarrah 2013 honeys with methylglyoxal levels ranging from 137.0 to 145.5 mg/kg. The remaining honeys contained less than 100 mg/kg methylglyoxal. Phytochemicals, including methylglyoxal, remain largely uncharacterised in Western Australian honeys. Concentrations of methylglyoxal previously found in Jarrah honey were 10–18 mg/kg for four samples and 120 mg/kg for a fifth sample, determined by HPLC–mass spectroscopy (Dawes and Dall 2014). In the same study, five samples each of three other eucalypt honeys were examined, and methylglyoxal levels of 15–22 mg/kg were found (Dawes and Dall 2014), indicating overall relatively low levels of methylglyoxal in eucalypt honeys. The majority of these values are lower than those obtained in the current study, possibly due to methodological differences or differences between honey samples. A recent study tested 35 samples of Australian *Leptospermum polygalifolium* honey for methylglyoxal content by infrared spectroscopy and found a range of 279–1755 mg/kg, with an average of 604 mg/kg (Sultanbawa et al. 2015). They also tested 74 other Australian honeys and found a lower range, with the smallest value being 8 mg/kg (Sultanbawa et al. 2015). The range of values obtained for each physicochemical parameter are in keeping with the natural variation seen in honey and also confirm that non-*Leptospermum* honeys as a generalisation do not have noteworthy levels of methylglyoxal.

Hydrogen peroxide levels accumulated in honeys after 2 h ranged from 22.7 to 295.5 μ mol and were highest for

Table 2 Antimicrobial activity of honeys against *S. aureus* ATCC 25923, determined by agar well diffusion and phenol equivalence assay (mean $\pm$ SD)

Honey	Zone of inhibition (mm) 50% w/v honey ^a	Phenol equivalence 25% w/v (filtered) honey solution	
		Total activity ^b	Non-peroxide activity
Banksia	17.9 $\pm$ 0.7	14.8 $\pm$ 1.5	–
Blackbutt	20.0 $\pm$ 0.3	22.5 $\pm$ 0.3	–
Bottlebrush	13.5 $\pm$ 1.6	11.4 $\pm$ 0.9	–
Jarrah (2015)	18.6 $\pm$ 1.3	19.2 $\pm$ 1.3	–
Jarrah (2013)	13.3 $\pm$ 1.0	–	–
Marri	15.3 $\pm$ 1.1	10.5 $\pm$ 1.1	–
Moort	19.7 $\pm$ 1.2	–	–
Spring	16.5 $\pm$ 0.4	17.0 $\pm$ 0.1	–
WA manuka	–	10.2 $\pm$ 0.4	10.1 $\pm$ 0.5
Wandoo	18.3 $\pm$ 0.7	10.9 $\pm$ 0.7	–
Activon	–	–	–
Capilano	–	–	–

– No activity or no zone

^a Honey solutions were unfiltered

^b Total activity was determined in the absence of catalase, and non-peroxide activity was determined after treatment with catalase

the four honeys Jarrah 2015, Moort, Wandoo and Spring, which were all above 200 μmol . The remaining honeys showed substantially lower H_2O_2 concentrations after 2 h of less than 100 μmol with Banksia honey having the lowest. Levels of H_2O_2 differed significantly between honeys at 2 h ($p < 0.05$). After 24 h, H_2O_2 concentrations had decreased in all honeys to 11.53 μmol or lower. Previous studies show that the accumulation of hydrogen peroxide after dilution of honey with water varies with honey concentration and is optimal at around 30% (Bang et al. 2003; Brudzynski et al. 2011). Hydrogen peroxide generation also varies over time, with an accumulation phase followed by a degradation phase (Brudzynski et al. 2011). This means that data from different studies may not be directly comparable as the honey concentration evaluated and time of accumulation may differ. Chen et al. (2012) found hydrogen peroxide levels in Australian honeys diluted to 25% w/v ranging from 0 to 1017 μmol after an undisclosed accumulation period (Chen et al. 2012), and to the best of our knowledge, there are no other published values for hydrogen peroxide levels accumulated in Australian honeys. Levels in the two *Leptospermum* honeys were relatively low, which is consistent with the theory that honeys with higher methylglyoxal concentrations tend to have lower hydrogen peroxide levels (Majtan et al. 2014).

All honeys with the exception of Activon contained endemic microbes and as such were sterilised by filtration for most antimicrobial activity assays. Screening of honeys at 50% (w/v) for activity by agar well diffusion showed zones of inhibition for 9 of the 12 honeys with zones ranging from 13.3 to 20.0 mm (Table 2). WA manuka, Activon and Capilano honeys did not show zones. Filtration had no significant effect on inhibition zone sizes for 50% w/v solutions of the two honeys Blackbutt or Jarrah 2015, with zones for Blackbutt honey of 20.0 mm (unfiltered) and 19.1 mm (filtered) ($p = 0.423$) and for Jarrah 2015 honey of 18.6 mm (unfiltered) and 17.2 (filtered) ($p = 0.172$).

The phenol equivalence (PE) assay, where honey is diluted to 25% (w/v), showed activity for 8 of the 12 honeys in the absence of catalase treatment, which is also termed “total activity” (Table 2). Blackbutt honey showed the highest total activity of 22.5% (w/v) PE, followed by Jarrah 2015 honey with 19.2% (w/v) PE. WA manuka honey had the lowest total activity of 10.2% (w/v) PE. The only honey to show non-peroxide activity was WA manuka honey, and PE values did not differ significantly with or without catalase treatment ($p = 0.682$). No activity was observed for Jarrah 2013, Moort, Activon and Capilano honeys either with or without catalase treatment by the PE method. The phenol equivalence assay was used by Irish et al. (2011) to examine the activity of a 92 Western Australian honey samples. In addition to activity for Jarrah and Marri honeys, they also found activity for a single

Table 3 Susceptibility of microorganisms to honeys (% w/v) determined by the broth microdilution method

	<i>S. aureus</i> ATCC 29213		<i>S. aureus</i> ATCC 25923		<i>S. aureus</i> NCTC 10442		<i>S. epidermidis</i> ATCC 11047		<i>E. faecalis</i> ATCC 29212		<i>E. faecalis</i> ATCC 51299		<i>A. baumannii</i> ATCC 7844		<i>E. coli</i> ATCC 25922		<i>P. aeruginosa</i> ATCC 27853		<i>S. Typhimurium</i> ATCC 13311		MIC geometric mean
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	
Banksia	8	16	8	8	8	8	8	8	>32	>32	32	>32	16	16	16	16	32	32	32	32	16.0
Blackbutt	8	8	8	8	8	8	4	4	32	32	32	32	16	16	16	16	16	16	16	16	13.0
Bottlebrush	8	8	8	16	8	16	16	16	32	>32	>32	>32	16	16	16	16	32	32	32	32	18.4
Jarrah (2015)	8	8	16	16	16	16	16	16	32	32	16	32	16	16	16	16	16	16	16	16	16.0
Jarrah (2013)	16	16	16	32	32	32	32	32	>32	>32	32	>32	32	32	32	32	32	32	32	32	27.9
Marri	16	16	16	16	16	16	16	16	32	>32	32	>32	32	32	32	32	32	32	32	32	24.3
Moort	16	16	4	4	16	32	4	4	32	>32	>32	>32	32	32	32	32	32	32	32	32	18.4
Spring 2014	8	8	16	16	16	16	16	16	32	32	32	32	16	16	16	16	32	32	32	32	19.7
WA Manuka	16	16	16	16	16	16	16	16	32	>32	>32	>32	16	32	16	32	32	16	32	32	19.7
Wandoo	8	8	16	16	16	16	16	16	32	>32	>32	>32	16	32	32	32	32	32	32	32	19.7
Activon	16	32	16	16	16	32	16	32	32	>32	>32	>32	32	32	32	32	32	32	16	32	22.6
Capilano	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	64.0
MIC geometric mean	12.7	12.7	12.7	12.7	12.7	12.7	14.3	14.3	35.9	35.9	33.9	33.9	22.6	22.6	24.0	24.0	28.5	28.5	26.9	26.9	

Table 4 Effect of catalase on the antimicrobial activity of honey against *S. aureus* ATCC 25923

Honey		Treatment		Fold change ^a	<i>p</i> value [*]
		None	+Catalase		
Banksia	MIC	8	32	4	0.013
	MBC	16	>32	>2	0.038
Blackbutt	MIC	8	32	4	0.001
	MBC	8	>32	>4	0.001
Bottlebrush	MIC	8	32	4	0.013
	MBC	16	>32	>2	0.038
Jarrah (2015)	MIC	16	32	2	0.057
	MBC	16	>32	>2	0.038
Jarrah (2013)	MIC	32	>32	≥2	0.030
	MBC	32	>32	≥2	0.029
Marri	MIC	16	>32	>2	0.019
	MBC	16	>32	>2	0.019
Moort	MIC	8	>32	>4	0.015
	MBC	8	>32	>4	0.015
Spring	MIC	16	>32	>2	0.019
	MBC	16	>32	>2	0.019
WA manuka	MIC	16	16	0	1.000
	MBC	16	16	0	0.649
Wandoo	MIC	16	>32	>2	0.019
	MBC	32	>32	≥2	0.057
Activon	MIC	16	16	0	0.101
	MBC	16	16	0	0.723
Capilano	MIC	>32	>32	0	—
	MBC	>32	>32	0	—

— No value

* MICs and MBCs for each honey were analysed by Student's two-tailed t-test, assuming unequal variance. *p* values (<0.05) show significant differences in antimicrobial activity between each catalase-treated and untreated honey, and significant values are indicated in bold type

^a Fold change in MIC and MBC after catalase treatment

Blackbutt honey sample, a single Moort honey sample, one out of five Banksia honey samples and two out of seven Wandoo honey samples. These results are largely in agreement with those from the current study, and any differences are likely due to natural variation between samples.

Honeys also showed a range of antimicrobial activity by broth microdilution (Table 3). To obtain a single measure of all MICs, the geometric mean of the MIC and MBC was calculated for each honey and for each test organism. Blackbutt honey had the lowest geometric mean MIC (13.0%), followed by Jarrah 2015 and Banksia honeys, both with geometric mean MICs of 16.0%. Blackbutt honey also showed the lowest geometric mean MBC (13.0%), followed by Jarrah 2015 honey (17.1%). The remaining honeys showed geometric mean MICs ranging

Table 5 Log reduction values for *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 after treatment with 40% (w/v) honey (mean ± SD)

Honey	<i>S. aureus</i> ATCC 25923		<i>E. coli</i> ATCC 25922	
	1 h	2 h	1 h	2 h
Banksia	1.4 ± 0.2	2.8 ± 0.4	0.8 ± 0.3	1.7 ± 0.4
Blackbutt	3.7 ± 0.2	— ^a	— ^a	— ^a
Bottlebrush	2.9 ± 0.7	—	0.7 ± 0.3	1.3 ± 0.2
Jarrah (2015)	—	—	2.6 ± 0.6	—
Jarrah (2013)	— ^a	—	1.4 ± 0.3	2.3 ± 0.8
Marri	—	—	1.9 ± 0.5	2.9 ± 0.2
Moort	— ^a	—	0.9 ± 0.2	1.6 ± 1.0
Spring	— ^a	—	1.9 ± 0.1	— ^a
WA manuka	3.1 ± 0.2	— ^a	1.3 ± 0.2	1.5 ± 0.7
Wandoo	2.9 ± 0.1	3.4 ± 0.4	1.6 ± 0.3	2.5 ± 0.7
Activon	— ^a	—	2.7 ± 1.3	— ^a
Capilano	1.4 ± 0.2	— ^a	0.7 ± 0.4	0.7 ± 0.5
Control (0.85% saline)	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.3	0.4 ± 0.2

— Organisms not detected (below the limit of detection of 2×10^3 cfu/ml)

^a Low numbers of organisms detected in one of the three experimental repeats only

from 18.4 to 27.9% (w/v), and MBC geometric means ranging from 21.1 to 34.3% (w/v). Capilano honey was the least active and did not inhibit any organism at the highest concentration of 32%. Analysis of log₂ MIC values by one-way ANOVA confirmed that honeys differed significantly in activity (*p* < 0.0001) and that MICs for all honeys were significantly lower than those of Capilano honey. Furthermore, there were no significant differences between the MICs of the remaining eleven honeys, including Activon (*p* > 0.05).

For test organisms, geometric mean MICs ranged from 12.7% for *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 to 35.9% for *E. faecalis*. Geometric mean MBCs ranged from 14.3% for *S. aureus* ATCC 29213 to 53.8% for *E. faecalis* (Table 3). Six of the honeys inhibited the growth of *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 at 8% with Moort honey showing inhibition at 4%. Furthermore, Blackbutt and Moort honeys inhibited *S. epidermidis* at 4% compared to Activon honey which had a higher MIC of 16%. Both *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 (VRE) showed the least susceptibility to the honeys with MICs of 32% or higher, which were significantly higher than MICs obtained for the remaining Gram-positive bacteria (*p* < 0.05). For the four Gram-negative bacteria, geometric mean MICs ranged from 22.6 to 28.5% and did not differ significantly (*p* > 0.05).

Removal of hydrogen peroxide by the addition of catalase enzyme did not change the MICs of either Activon

or WA manuka honey (Table 4), suggesting that hydrogen peroxide makes a negligible contribution to total activity in these two *Leptospermum* honeys. For the remaining 10 honeys, 8 showed statistically significant decreases in activity following catalase treatment (p value range 0.001–0.030) with MICs increasing at least fourfold. For Jarrah 2015 honey the difference in MIC was only twofold (not significant; $p = 0.057$) and Capilano honey did not show activity with or without catalase. Whilst hydrogen peroxide plays a role in the activity of honey (Brudzynski et al. 2011), in the current study there was no obvious relationship between level of hydrogen peroxide and antibacterial activity by any method. Those honeys with the highest hydrogen peroxide were not the most active honeys, which also suggests that low hydrogen peroxide honeys may contain phytochemicals other than methylglyoxal that make an important contribution to overall activity.

The time-kill assay demonstrated that treatment with most honeys resulted in substantial reductions in viability for both *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Table 5). After 1 h, *S. aureus* was not recovered from six of the honeys and for the remaining six honeys, log reduction values ranged from 1.42 to 3.71. After 2 h, *S. aureus* was only recovered from Banksia and Wandoo honeys. In contrast, honey showed comparatively less activity against *E. coli*, with organisms recovered from all honeys after 1 h with the exception of Blackbutt. After 2 h, *E. coli* was not recovered from a total of four honeys and log reductions for the remaining eight honeys ranged from 0.68 to 2.93. Log reductions for the controls (in 0.85% saline) after 2 h were 0.1 for *S. aureus* and 0.3 for *E. coli*.

The current study shows a range of antimicrobial activity for the ten Western Australian honeys. The two most active honeys in the zone of inhibition assay were Blackbutt and Moort, whereas in the phenol equivalence assay, Blackbutt and Jarrah (2015) were the most active. The lowest geometric mean MIC was obtained for Blackbutt honey, followed by Jarrah (2015) and Banksia. Taken together, these data suggest that Blackbutt was the most active honey. There is no obvious explanation for the high activity from the physicochemical data, suggesting that factors other than those measured contribute to activity. In addition to data from Irish et al. (2011) on Western Australian honeys described above, research from the Western Australian Department of Agriculture and Food also found that Marri and Jarrah honeys had high average total activity values of 31.1% (from 29 samples) and 30.2% (from 69 honey samples) (Manning, unpublished data). However, in our study, Marri was not overly active in the phenol equivalence assay for total activity. Data from our study are broadly in agreement with previous results, with 8 out of 10 WA honeys showing total activity by the phenol equivalence assay and only one

honey (WA manuka) demonstrating non-peroxide activity. However, comparisons with previous studies are limited since little other data are available and previous data also indicate high variability in antimicrobial activity amongst different honeys derived from the same floral source.

Evaluation of antimicrobial data obtained by each of the different methods revealed inconsistencies, suggesting that results are strongly influenced by the particular method used. As an example, the six honeys Activon, Jarrah 2015, Marri, Spring, WA manuka and Wandoo all had an MIC of 16 against *S. aureus* ATCC 25923, suggesting similar activity. In contrast, both WA manuka and Activon showed no activity in the 50% screening assay and only WA manuka showed non-peroxide activity in the phenol equivalence assay. This indicated that both Activon and WA manuka showed no or only poor activity in agar-based assays but showed activity comparable to most other honeys in the broth-based assay, in turn suggesting that the agar-based assays may not accurately reflect antimicrobial activity. One explanation is that these assays rely on the diffusion of antimicrobial substances through the agar, and not all hydrophobic or large molecules diffuse easily meaning that activity due to these molecules may be underestimated. Methodological considerations aside, it was unexpected that Activon honey did not show activity in either of the agar diffusion assays. The product states that it is medical-grade manuka honey but does not state the level of activity of the honey. If it was at the lower end of the activity spectrum it is possible that activity was diluted to below detection by agar diffusion; however, results of MIC tests and time-kill assays do not support this as it was highly active in these assays. It remains possible that both of the agar assays were not conducted correctly, but this does not seem likely given that the remaining honeys showed activity in these assays. At this stage, there is no likely explanation for why the Activon honey did not show activity in both agar-based assays.

Honey, based on historical use and current scientific research, is re-emerging as a potential treatment for a number of conditions. The development of any medicinal product, regardless of whether it is derived from a natural source or synthesised, relies on a body of sound laboratory and clinical studies to support its use. Whilst a reasonable amount of research has been conducted on manuka honey from *Leptospermum scoparium* (Jenkins et al. 2011; Lu et al. 2013), native to New Zealand, very little data have been published on the antibacterial activity of Western Australian honeys.

Honey has garnered significant interest as a potential treatment for wounds. However, only a limited number of specific honeys have been commercialised and developed into sterile products suitable for clinical use. These

include manuka honey from the floral source *Leptospermum scoparium* (New Zealand), Jellybush from *Leptospermum polygalifolium* (Australia), Revamil (Netherlands) and Surgihoney™ (United Kingdom) which is a modified honey. Honeys with a *Leptospermum* floral source are deemed highly desirable because the non-peroxide activity is thought to be longer lasting in a wound environment than peroxide-based activity, which is quenched by catalase (Cooper et al. 1999; Majtan and Majtan 2010). However, two of the above-named honeys, Revamil and Surgihoney™ have hydrogen peroxide-based activity, suggesting that both peroxide and non-peroxide honeys may be clinically effective.

Whilst numerous in vitro studies, including the current study, show that different honeys vary in antibacterial activity (Irish et al. 2011; Chen et al. 2012), what remains to be shown is whether these differences are clinically relevant. The majority of clinical studies with honey compare a single honey to a conventional treatment and only a few compare more than one honey. Lusby et al. (2006) compared the effects of lavender honey, Medihoney, lavender oil, canola oil and an untreated control on the healing of experimental wounds in rats (Lusby et al. 2006). By day 12, there were no significant differences between treatments for wound size with all wounds significantly healed. Wounds treated with lavender honey and Medihoney had significantly lower capillary volume than the remaining treatments, and the two honey treatments did not differ significantly. Haryanto et al. (2012) compared Indonesian honey (produced by *Apis dorsata*) to manuka honey for healing experimental wounds in mice and found no significant differences (Haryanto et al. 2012). Nisbet et al. (2010) investigated chestnut honey, rhododendron honey and blossom (multifloral) honey for wound healing in rabbits and found no significant differences between honeys (Nisbet et al. 2010). Although these three animal studies do not provide conclusive evidence, they collectively support the notion that a range of honeys are likely to be clinically useful rather than only a select few.

In summary, examination of the antimicrobial activity of a number of unique Western Australian honeys showed that most had antibacterial activity, although the degree of activity varied depending on which method was used. Importantly, there was variation in the extent of activity obtained using each antimicrobial assay, providing further evidence that values obtained for antimicrobial activity are heavily influenced by the test methodology. A major limitation of this study was the relatively small number of honey samples examined, and further work is therefore required to better understand the relationship between chemical composition and bioactivity, to determine optimal antibacterial testing assay conditions and lastly to determine the potential clinical usefulness of the honeys.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest to declare.

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