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

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Running headline: Natural products for the treatment of *Clostridium difficile* infection

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Abstract

Aims: To investigate the antimicrobial activity of various natural products against *C. 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 (FIC) 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.

Key words: garlic, cinnamon, peppermint oil, alternative therapy, CDI

Introduction

Clostridium difficile is a Gram-positive, spore forming anaerobic bacterium that causes disease ranging from antibiotic-associated diarrhea to life threatening pseudomembranous colitis (McDonald *et al*., 2005). *C. difficile* is one of the main causes of nosocomial diarrhea 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 hyper-virulent 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 (Henrich *et al*., 2009, Owens *et al*., 2008). The usual treatment for CDI is antibiotics which are 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

Microorganisms

Organisms were obtained from the School of Pathology and Laboratory Medicine 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).

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 ^{o} 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 µm 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 pre-reduced *Brucella* agar supplemented with 5 µg hemin, 1 µg vitamin K_1 and 5% (v/v) laked sheep blood. Plates were incubated for 24-48 h at 35 ^{o}C in an anaerobic chamber (Don Whitley Scientific), while aerobic bacteria were cultured on blood agar for 18-24 h at 35^{o}C. Growth was suspended in pre-reduced 0.85% saline for anaerobic bacteria and non-reduced 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 ml^{-1} for *C. difficile* based on

preliminary data and Clinical and Laboratory Standards Institute (CLSI) guidelines, and 1×10^8 cfu ml^{-1} 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 (Table 1 and 2). Pre-reduced *Brucella* agar or MHA was lawn inoculated with the inocula. Wells (8 mm) were cut into each inoculated agar plate and a 100 µl 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 millimeter.

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, 2015, Clinical and Laboratory Standards Institute, 2009). Briefly, each product was serially diluted two-fold across a 96-well tray. The final inocula concentration for anaerobes and aerobes was approximately 1×10^6 cfu ml^{-1} and 5×10^5 cfu ml^{-1}, 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 sub-culturing 10 µl aliquots from non-turbid 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 two-fold dilutions of 20% DMSO were prepared and tested against all the microorganisms 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 microtiter 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: $FIC_{antibiotic} = MIC_{antibiotic\ combination}/MIC_{antibiotic\ alone}$, $FIC_{natural\ product} = MIC_{natural\ product\ combination}/MIC_{natural\ product\ alone}$ and FIC index= $FIC_{index} = FIC_{antibiotic} + FIC_{natural\ product.}$ 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 2-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 (Table 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 microorganisms (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 with 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 allicin, *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 two-fold dilutions of 20% DMSO were tested against all the microorganisms. 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 flora of the gastrointestinal tract (Table 5 and 6). It showed that lactic acid bacteria such as *Lactobacillus 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 4-fold 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 checkboard 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 (Figure 1). When *trans*-cinnamaldehyde was combined with vancomycin, there was at least a 4-fold increase in inhibitory effect of vancomycin and a 2-fold 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 2-fold 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 hyper-virulent *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* 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). Allicin is generated by crushing garlic cloves, which results in the conversion of alliin to allicin and other thiosulfinates 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 (Valgas *et al*., 2007, Hammer *et al*., 1999). However, factors other than the susceptibility of the microorganism 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 (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 4-fold higher against the four *C. difficile* isolates than those obtained in Hammond and Donkor (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 microorganisms 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 (Rea *et al*., 2011, Edlund *et al*., 1997, Al-Nassir *et al*., 2008). For example, Edlund and Barkholt (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, *Pediococcus* species, and lactobacilli (Rea *et al*., 2011, Edlund *et al*., 1997). 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 distruptive to the commensal flora. *L. 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 microorganisms 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 (Watanakunakorn, 1984, Hancock and Fitz-James, 1964). On the other hand, metronidazole acts on susceptible microorganisms 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 recognised as safe (GRAS) 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 Organization, 2004). Some of the adverse events that may arise from the use of medicinal plants are due to 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 pre-clinical 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 Organization, 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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Figures:

Figure 1. Isobolograms representing the effect of *trans*-cinnamaldehyde on antibiotics against *C. difficile*.

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

Raw product (juice or dried powder)	solvent	Mean zone of inhibition in mm (± SD)* *S. aureus* ATCC 29213	*E. coli* ATCC 25922	*C. perfringens* ATCC 13124	*C. difficile* ATCC 13366	*C. difficile* ribotype UK 014	*C. difficile* ATCC 700057
Onion juice (100% v/v)	-	10.7 ± 0.6	-	15.3 ± 0.6	10.3 ± 0.6	-	11.0 ± 0.0
Garlic juice (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
Ginger juice (100% v/v)	-	-	-	-	-	-	-
WA Manuka honey (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 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 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 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 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
Trimethoprim 5mg/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

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. *** P < 0.001
If no zone of inhibition was observed, an eight 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 *C. difficile* isolates and three comparator isolates.

Processed product (capsules, pills, oils and gels)	solvent	Mean zone of inhibition in mm (± SD)*					
		S. aureus **ATCC 29213**	***E. coli*** **ATCC 25922**	***C. perfringens*** **ATCC 13124**	***C. difficile*** **ATCC 13366**	***C. difficile*** **ribotype UK 014**	***C. difficile*** **ATCC 700057**
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; 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.

Table 3. Susceptibility of microorganisms to raw natural products determined by the broth microdilution method.

Raw product		solvent	Microorganisms						
			S. aureus **ATCC 29213**	***E. coli*** **ATCC 25922**	***C. perfringens*** **ATCC 13124**	***C. difficile*** **ATCC 13366**	***C. difficile*** **ribotype UK 014**	***C. difficile*** **ATCC 43598**	***C. difficile*** **ATCC 700057**
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%	4.7	4.7	9.4	9.4	9.4	9.4	9.4
	MBC	DMSO	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%	75	150	>150	>150	>150	>150	>150
	MBC	DMSO	150	150	>150	>150	>150	>150	>150
Cinnamon powder (mg ml^{-1})	MIC	SDW	37.5	>150	>150	>150	150	>150	150
	MBC		>150	>150	>150	>150	>150	>150	>150
	MIC	20%	37.5	150	75	75	75	75	75
	MBC	DMSO	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%	>150	>150	>150	>150	>150	>150	>150
	MBC	DMSO	>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 microorganisms to processed natural products by broth microdilution (20% DMSO was used as the primary solvent).

Processed products		Microorganisms						
		S. aureus **ATCC 29213**	***E. coli*** **ATCC 25922**	***C. perfringens*** **ATCC 13124**	***C. difficile*** **ATCC 13366**	***C. difficile*** **ribotype UK 014**	***C. difficile*** **ATCC 43598**	***C. difficile*** **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 capsule (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.

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

Raw products		solvent	Microorganisms: *B. fragilis* NCTC 9343	*B. vulgatus* NCTC 10583	*B. thetaiotaomicron* 5018	*Lactobacillus casei* Q328	*L. casei* 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		Microorganisms				
		B. fragilis **NCTC 9343**	***B. vulgatus*** **NCTC 10583**	***B. thetaiotaomicron*** **5018**	***Lactobacillus casei*** **Q328**	***L. casei*** **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.

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

Organism	Metronidazole			Vancomycin		
	Trans-cinn	MET	FIC	Trans-cinn	VAN	FIC
C. difficile ATCC 700057						
Trans-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**
C. difficile ATCC 13366						
Trans-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

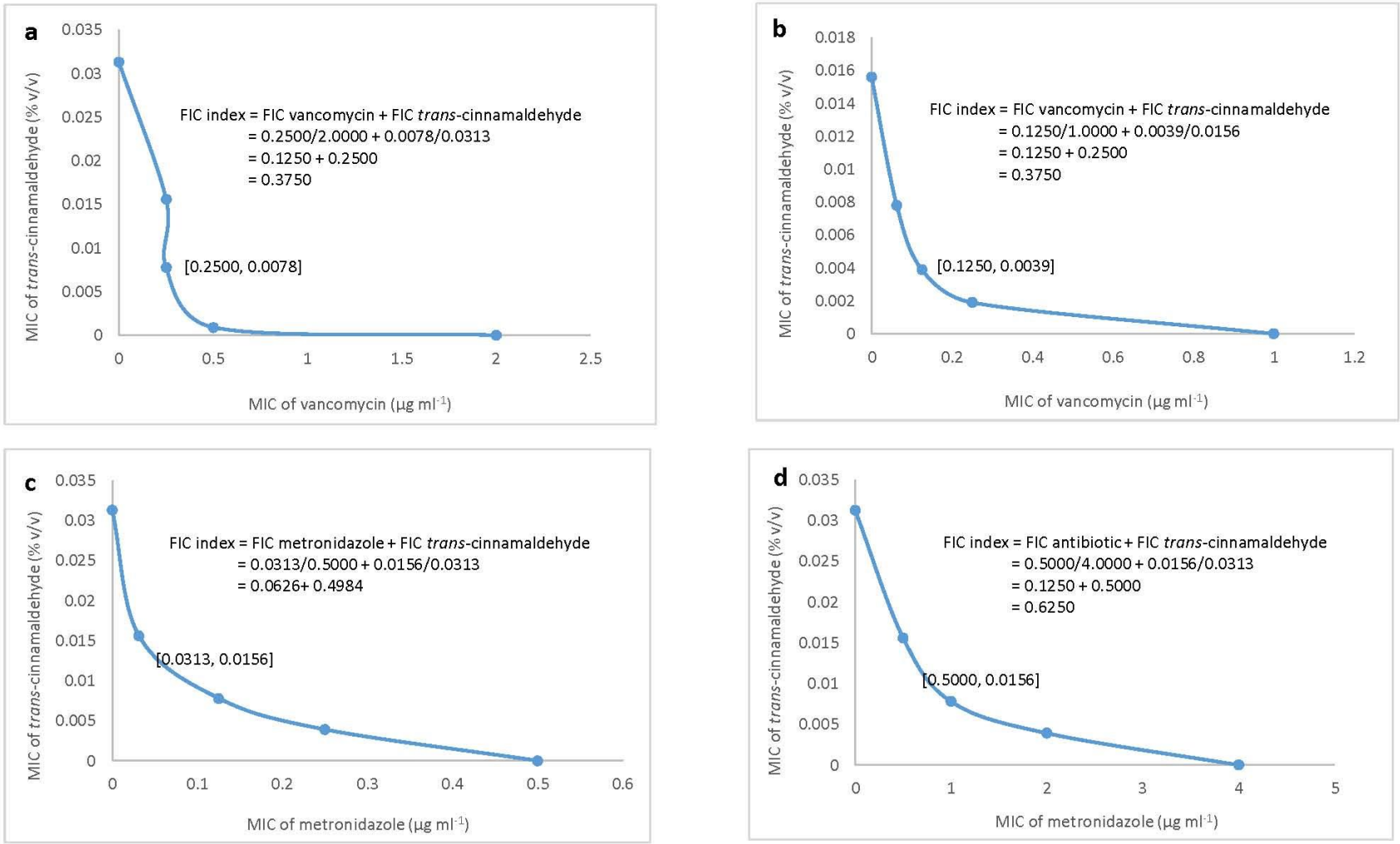

Figure 1. Isobolograms representing the effect of *trans*-cinnamaldehyde on antibiotics against *C. difficile*. a) *trans*-cinnamaldehyde and vancomycin against *C. difficile* ATCC 70057, b) *trans*-cinnamaldehyde and vancomycin against *C. difficile* UK 027 ATCC 13366, c) *trans*-cinnamaldehyde and metronidazole against *C. difficile* ATCC 70057, d) *trans*-cinnamaldehyde and metronidazole against *C. difficile* UK 027 ATCC 13366.