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

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INTRODUCTION

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus (Figure 1).[1] The main virulence factors are toxin A (TcdA), toxin B (TcdB) and binary toxin (CDT).[2] Typically, *C. difficile* infection (CDI) occurs following the disruption of normal enteric flora, usually post-antimicrobial exposure, leading to the proliferation and germination of *C. difficile* spores, resulting in toxin production in the intestine.[2,3]

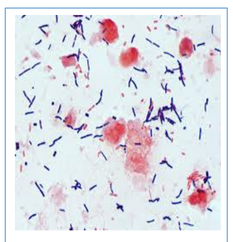


Figure 1. Gram stain of *C. difficile*

Clinical manifestations of CDI range from mild/self-limiting diarrhoea to fulminant colitis.[1] Conventional treatments are metronidazole and vancomycin.[1,4] Despite retaining good *in vitro* efficacy against *C. difficile,* several issues remain surrounding the use of these agents such as unacceptably high rates of CDI recurrence and reduced efficacy *in vitro*.[1]

OBJECTIVE

The purpose of this study was to investigate the effect of natural products on toxin production and activity in *C. difficile in vitro.*

METHODS

Twenty-two natural products were selected for testing, based on historical evidence, current popularity and feasibility, against three reference strains and one clinical isolate.

Assays:

1. Toxin protection activity: Vero and HT-29 cell cytotoxicity and neutral red uptake assays.[5]
2. The indirect effect of products on toxin-mediated cytotoxicity: Tissue culture using Vero and HT-29 cell lines (Figure 2).
3. Effect of treatments on *C. difficile* toxin production using ELISA (Figure 3).

Figure 2. Tissue culture

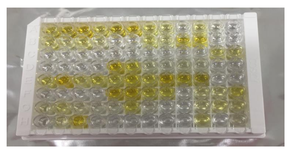


Figure 3. ELISA

CONCLUSIONS

This study highlights the activity of a number of food-grade and plant-derived products against *C. difficile* toxins *in vitro*.

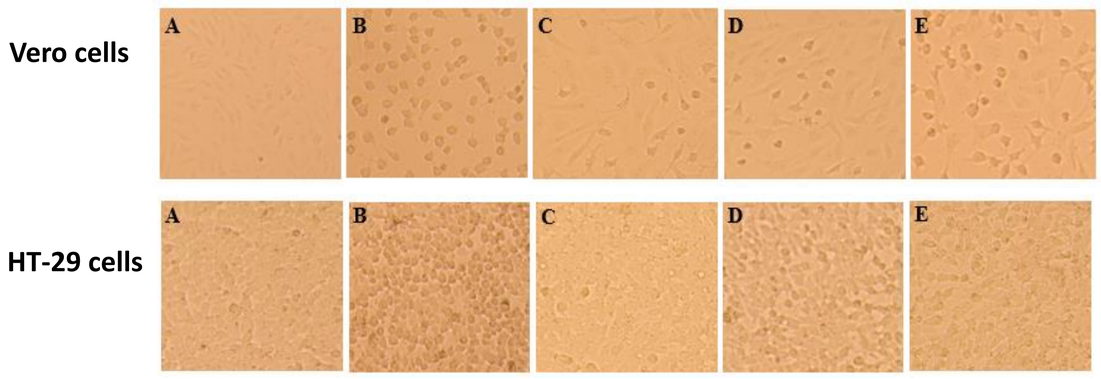
These results suggest that several natural products may have the potential to be considered as either alternative or complementary treatment options for CDI.

Applying anti-virulence strategies such as disruption of toxin production and toxin-mediated pathology in patients with CDI may be an effective approach in either controlling or treating infection.

Studies are required to further investigate the potential benefits of these natural products *in vivo*.

RESULTS

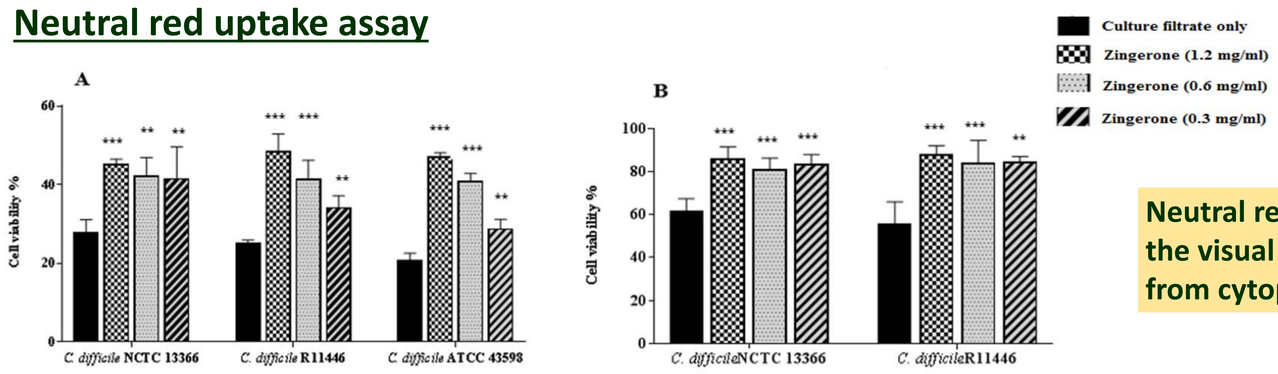
Toxin protection assay



Zingerone (≥ 0.3 mg/ml) protected both cell lines from cytopathic effects of toxin either when culture filtrates were incubated for 2 h with zingerone or when cells were pre-incubated with zingerone prior to the addition of culture filtrates.

Figure 4. Protection from cytopathic effect on Vero and 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 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 × 40.

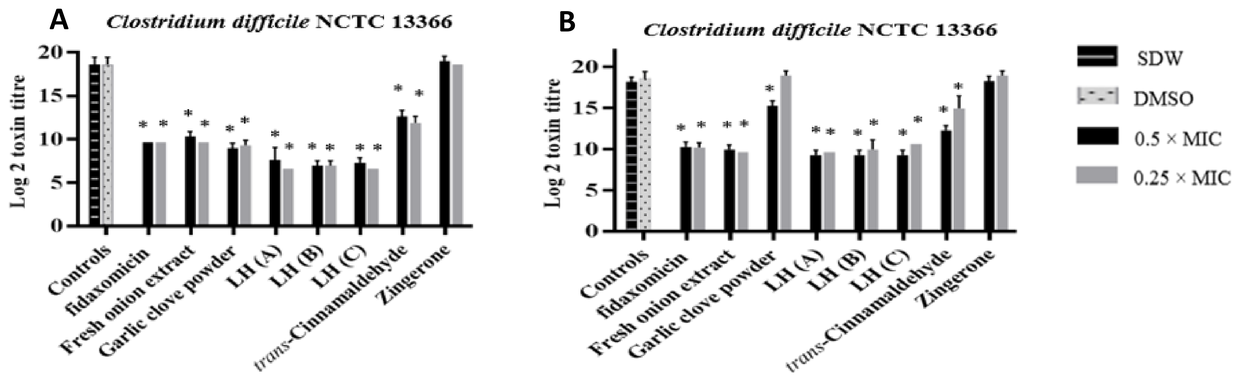
Neutral red uptake assay



Neutral red uptake assay confirmed the visual perception of protection from cytopathic effect.

Figure 5. 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.

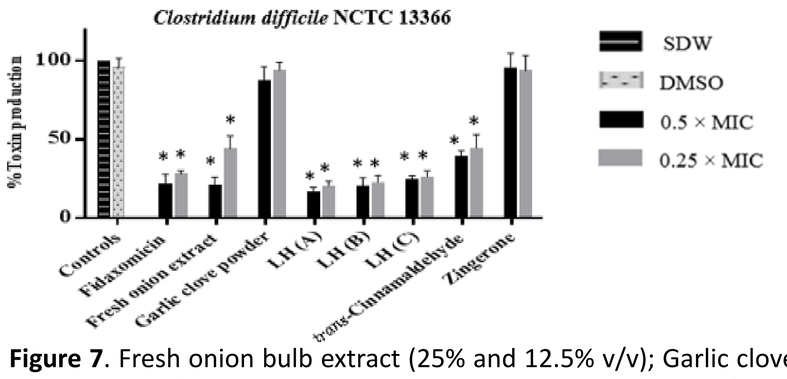
Indirect effect of treatments on *C. difficile* cytotoxicity



Three *Leptospermum* honeys (4% w/v), fresh onion bulb extract (12.5% v/v), garlic clove powder (4.7 mg/ml) and *trans*-cinnamaldehyde (0.005% v/v) reduced the cytotoxicity titre.

Figure 6. 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.

Effect of treatments on *C. difficile* toxin production



Three *Leptospermum* honeys (4% w/v), fresh onion bulb extract (12.5% v/v) and *trans*-cinnamaldehyde (0.005% v/v)reduced toxin production.

Figure 7. 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: ▲= $P < 0.05$, ° = $P < 0.01$, * = $P < 0.001$ compared to controls. *LH, *Leptospermum* honey; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

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