



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

Niloufar Roshan^a, Thomas V. Riley^{a,d}, Daniel R. Knight^c, Katherine A. Hammer^a

^a School of Biomedical Sciences, The University of Western Australia, Western Australia.

^b PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Western Australia.

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

^d School of Medical & Health Sciences, Edith Cowan University, Western Australia.



INTRODUCTION

Clostridium difficile 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}

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

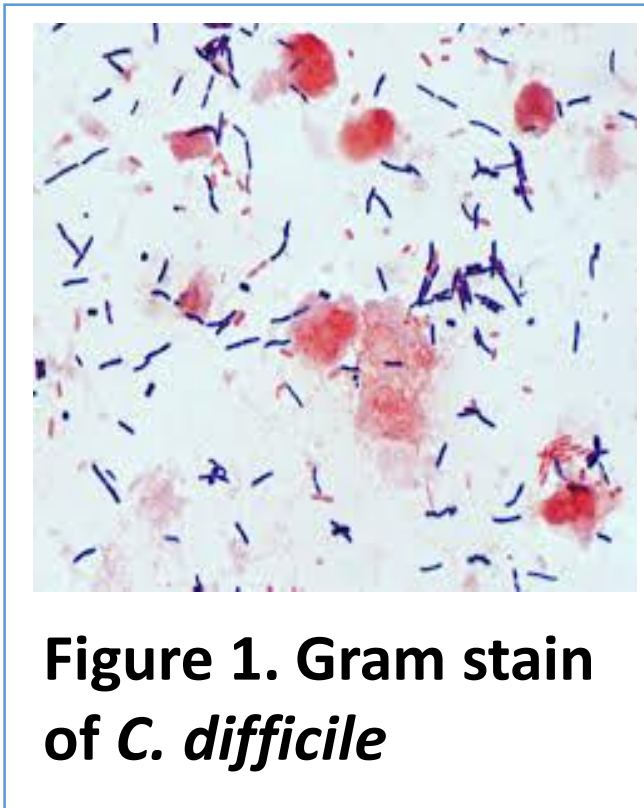


Figure 1. Gram stain of *C. difficile*

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).

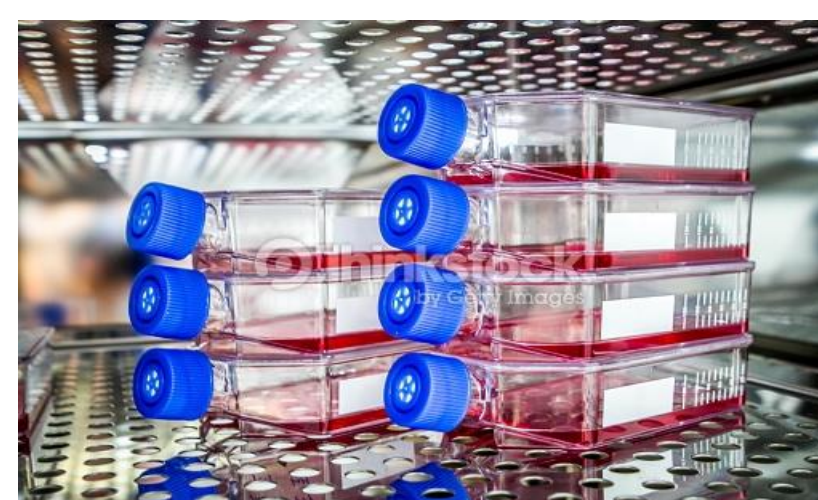


Figure 2. Tissue culture

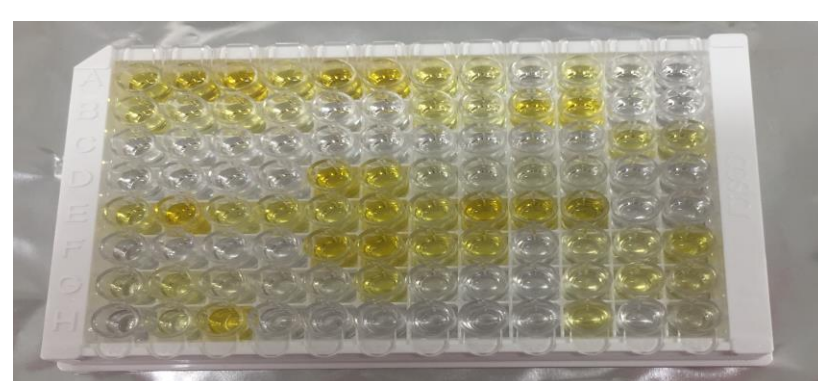


Figure 3. ELISA

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

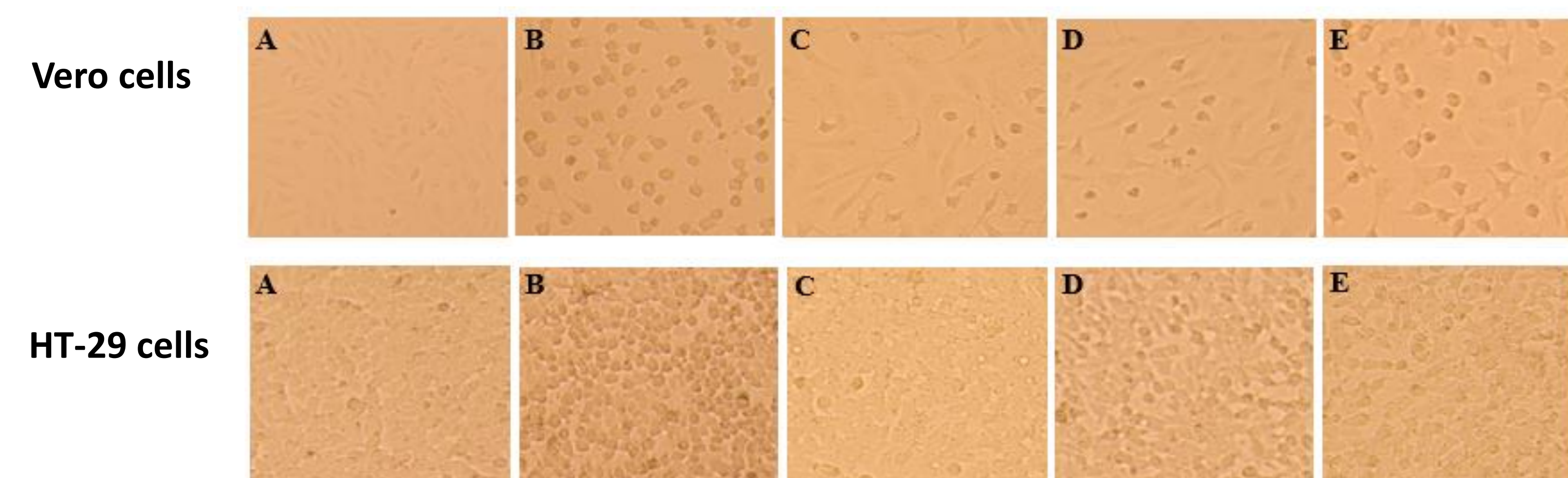


Figure 4. Protection from cytopathic effect on Vero and HT-29 cells using microscopy (*C. difficile* NCTC 13366 culture filtrate and zingerone were incubated at 37 °C for 2 h prior to being added to Vero cell monolayers). A: no culture filtrate; B: culture filtrate only; C: zingerone (1.2 mg/ml); D: zingerone (0.6 mg/ml); E: zingerone (0.3 mg/ml); Light microscopy × 40.

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.

Neutral red uptake assay

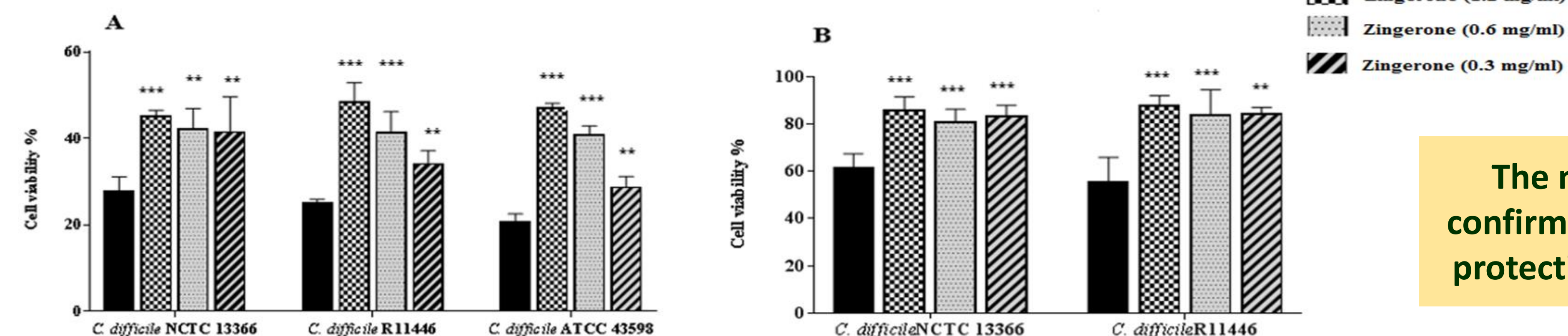


Figure 5. Cell viability determined by neutral red uptake assay. (Culture filtrates and treatments were incubated 2 h prior to being added to the cells); A: Vero cells; B: HT-29 cells. Statistical significance: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared to culture filtrate control.

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

Indirect effect of treatments on *C. difficile* cytotoxicity

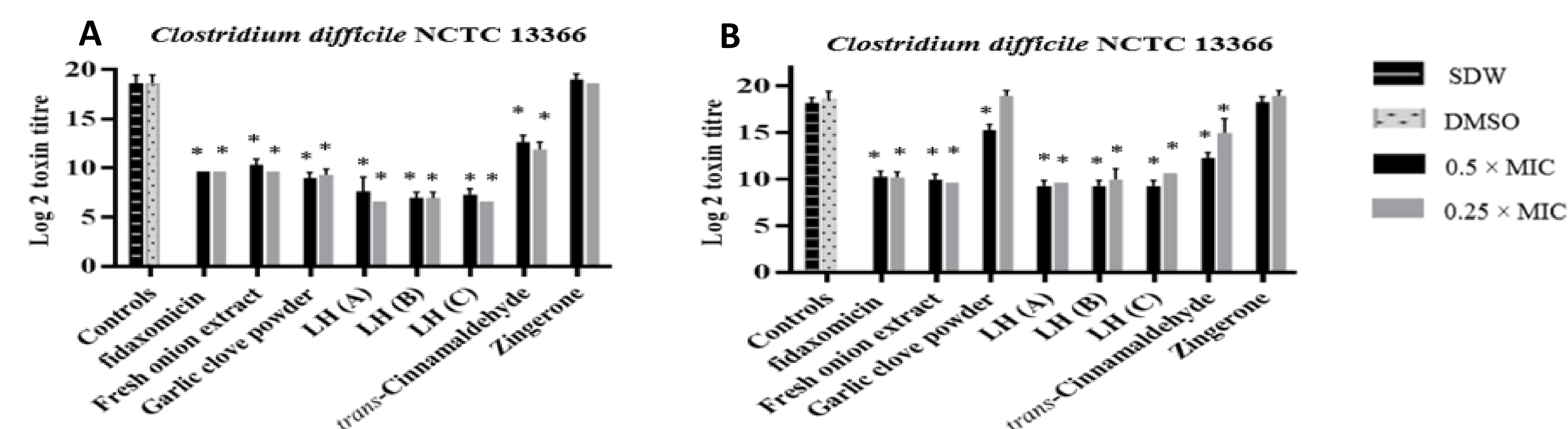


Figure 6. Indirect effect of treatments on *C. difficile* cytotoxicity on (A) Vero cells and (B) HT-29 cells. Concentrations of agents used in the assay: Fresh onion bulb extract (25% and 12.5% v/v); Garlic clove powder (4.7 and 2.3 mg/ml); *Leptospermum* honey (A), (B) and (C) (8% and 4% v/v); *trans*-Cinnamaldehyde (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.

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.

Effect of treatments on *C. difficile* toxin production

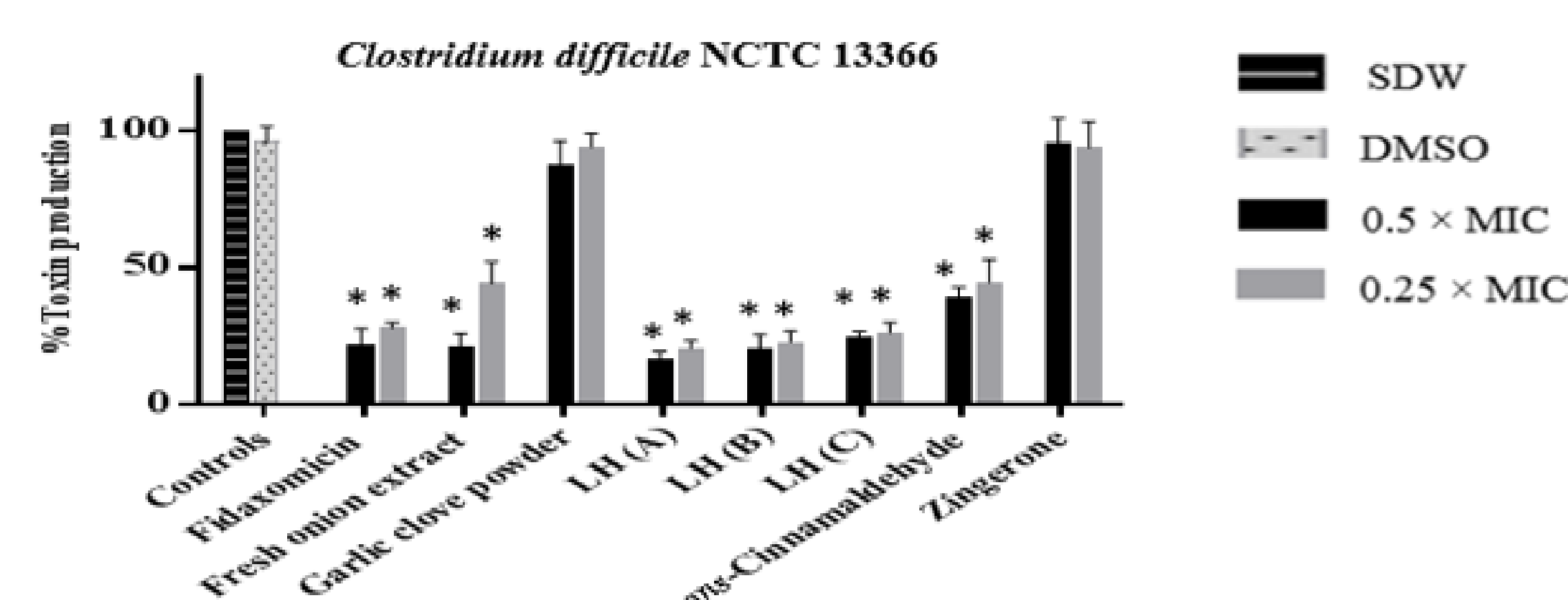


Figure 7. Fresh onion bulb extract (25% and 12.5% v/v); Garlic clove powder (4.7 and 2.3 mg/ml); *Leptospermum* honey (A), (B) and (C) (8% and 4% v/v); *trans*-Cinnamaldehyde (0.01% and 0.005 v/v); Zingerone (4.7 and 2.3 mg/ml); Fidaxomicin (0.06 µg/ml). Statistical significance: ▲ = $P < 0.05$, ° = $P < 0.01$, * = $P < 0.001$ compared to controls. *LH, *Leptospermum* honey; SDW, sterile distilled water; DMSO, dimethyl sulfoxide.

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

REFERENCES

1. Riley TV. From obscurity to superbug; The rise of *Clostridium difficile*. Healthcare Infection. 2010;15(3):59-61.
2. Paredes-Sabja D, Shen A, Sorg JA. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. Trends in microbiology. 2014;22(7):406-16.
3. Carter GP, Rood JI, Lyras D. The role of toxin A and toxin B in the virulence of *Clostridium difficile*. Trends in Microbiology. 2012;20(1):21-9.
4. Trubiano JA, Cheng AC, Korman TM, Roder C, Campbell A, May MLA, et al. Australasian society of infectious diseases updated guidelines for the management of *Clostridium difficile* infection in adults and children in Australia and New Zealand. Internal Medicine Journal. 2016;46(4):479-93.
5. Hinkson PL, Dinardo C, DeCiero D, Klinger JD, Barker RH. Tolevamer, an anionic polymer, neutralizes toxins produced by the BI/027 strains of *Clostridium difficile*. Antimicrobial Agents and Chemotherapy. 2008;52(6):2190-2195.

