

Pomegranate extract specifically inhibits *Clostridium difficile* growth and toxin production without disturbing the beneficial bacteria in vitro

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Objective: The aim of this study was to assess the pomegranate juice against the growth and toxin production of multidrug-resistant *Clostridium difficile* hypervirulent strain NAP1/027/BI and also against the growth of beneficial bacteria to prevent or suppress *C. difficile* infection (CDI).

Materials and methods: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were taken as parameters for the assessment of antimicrobial property of the pomegranate juice. Four different *C. difficile* hypervirulent strains NAP1/027/BI, *Lactococcus lactis* spp., *Lactobacillus casei*, and *Bifidobacterium animalis* were subjected to the broth dilution method to determine the MIC and MBC. Enzyme-linked immunosorbent assay (ELISA) was performed to determine clostridial toxin B (TcdB) production in the presence of pomegranate juice.

Results: The MIC and MBC of pomegranate juice containing punicalagin were found to be 390 µg/mL for all *C. difficile* hypervirulent strain NAP1/027/BI, and the growth of *L. lactis* spp., *L. casei*, and *B. animalis* was not inhibited. Pomegranate juice reduced TcdB production in *C. difficile* hypervirulent strain NAP1/027/BI.

Conclusion: This study highlights the potential of pomegranate juice to reduce CDI without affecting the beneficial bacteria. Pomegranate juice may be a useful antimicrobial agent to prevent or suppress CDI, avoiding the use of antibiotics.

Keywords: *Clostridium difficile*, pomegranate juice, antimicrobial agent, CDI

Introduction

Clostridium difficile is an important nosocomial pathogen. *C. difficile* infection (CDI) causes severe gastrointestinal disease in individuals undergoing treatment with antibiotics.¹ *C. difficile* produces two major toxins, namely, clostridial toxin A (TcdA) and clostridial toxin B (TcdB), which is responsible for toxin-mediated inflammation and disease. These toxins are mainly responsible for stimulation of an inflammatory response and cause damage to the mucosal epithelium in the gut.¹ Toxin B (TcdB) from ribotype 027/NAP1/BI is associated with an increased cytotoxicity, which leads to enhanced virulence.² *C. difficile* hypervirulent ribotype 027/NAP1/BI strain is associated with an increased morbidity and mortality.³ Vancomycin, fidaxomicin, and metronidazole are antibiotics currently used for the treatment of CDI. However, *C. difficile* strains resistant to metronidazole and reduced susceptibility to vancomycin have been reported in few studies.^{4,5} It is more difficult to treat *C. difficile* hypervirulent ribotype 027/NAP1/BI strain with fidaxomicin and vancomycin than other strains.⁶

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The development of multidrug-resistant bacteria is due to the prolonged use of antibiotics.⁶ This has significantly limited the efficacy of antibiotics. The increased virulence, degree of resistance, and recurrence of CDI have prompted the development of new antibacterial agents for the treatment of CDI.

Plants and extracts from plant materials have been used as antibacterial agents in traditional medicine worldwide.⁷ Pomegranate (*Punica granatum L.*) fruits have been used for nutritional and medicinal purposes for centuries. Pomegranate juice is rich in polyphenols. The major ellagitannin (ET) called punicalagin (2,3-hexahydroxy-diphenoyl-4,6-gallagylglucose) is the most abundant type of polyphenol present in the pomegranate juice.⁸ ETs belong to the chemical class of hydrolyzable tannins, which release ellagic acid (EA) on hydrolysis.⁸

A group of lactic acid bacteria (LAB) are called beneficial bacteria including probiotic strains of the *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* genera.⁹ These organisms are called probiotic organisms due to their beneficial roles in the gut, particularly essential in maintaining the intestinal microbial balance for inhibiting the growth of pathogenic microorganisms.¹⁰ The growth of beneficial bacteria, such as *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*, may therefore modulate gut microbiota. The healthy gut microbiota provides protection against numerous pathogens including *C. difficile*.¹¹ Susceptibility pattern of *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* to pomegranate juice is of interest in understanding the alteration of normal intestinal microflora when pomegranate juice is taken.

We hypothesized that pomegranate juice may reduce toxin production and also provide antibacterial effect against multidrug-resistant *C. difficile* hypervirulent strain NAP1/027/BI, without inhibiting the growth of some beneficial bacteria, which is advantageous in preventing CDI and recurrence. For this purpose, we studied the toxin production of *C. difficile* hypervirulent strain NAP1/027/BI in the presence of pomegranate juice and also studied the antibacterial effect of pomegranate juice against multidrug-resistant *C. difficile* hypervirulent strain NAP1/027/BI, *Lactococcus lactis* spp., *Lactobacillus casei*, and *Bifidobacterium animalis*.

Materials and methods

Bacterial strains

Four different *C. difficile* hypervirulent strains NAP1/027/BI, *L. lactis* spp., *L. casei*, and *B. animalis* were obtained from Routine Diagnostic, Institute for Medical Microbiology,

University Hospital Leipzig (Leipzig, Germany). All bacteria included in this study were again confirmed by matrix assisted laser desorption/ionization (VITEK MS; bioMérieux, France) according to manufacturer's instruction. All four *C. difficile* hypervirulent NAP1/027/BI strains were again confirmed by GeneXpert *C. difficile* PCR assay (Cepheid, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Composition of pomegranate juice

Pomegranate juice was brought from POM Wonderful (Los Angeles, CA, USA), which contains the following concentration of polyphenols: 1561 mg/L of punicalagins, 387 mg/L of anthocyanins, 121 mg/L of EAs, and 417 mg/L of other hydrolyzable tannins.

Determination of the minimum inhibitory concentration (MIC) of bacteria

According to European Committee on Antimicrobial Susceptibility, the MICs of pomegranate juice against different bacteria were determined in 96-well microtiter plate with round bottom. Different concentrations of pomegranate juice containing punicalagin ranging from 24.37 to 780 µg/mL were prepared in brain heart infusion broth for *C. difficile*, de Man Rogosa Sharpe broth for *L. casei* and *B. animalis*, and Mueller-Hinton broth for *L. lactis* spp. by serial dilutions. Then, 100 µL of standardized working bacterial suspension was inoculated into each column containing different concentrations of 100 µL of pomegranate juice and also to growth control (100 µL of medium), which provided the required final inoculum density of ~10⁵ CFU/mL. Afterward, the plates were incubated at 37°C for 48 hours at anaerobic condition for *C. difficile*, *L. casei*, and *B. animalis* and at 37°C for 24 hours in a CO₂ incubator for *L. lactis* spp. Inhibition of bacterial growth was determined visually. All the strains were tested in triplicate.

Determination of minimum bactericidal concentration (MBC)

MBC was determined by sub-culturing 10 µL of samples from each MIC well onto a fresh solid medium (brucella agar supplemented with 5% laked sheep blood and vitamin K1 [1 mg/L] for *B. animalis* and *C. difficile*, de Man Rogosa Sharpe agar for *L. casei*, and Trypticase soy agar with 5% sheep blood for *L. lactis* spp.), and the plates were incubated at appropriate temperature and condition for specific time. The highest dilution that yielded no single colony of bacteria was considered as MBC.

Determination of time period for antimicrobial activity

To determine the time period required for antimicrobial activity of pomegranate juice against *C. difficile*, the concentration of pomegranate juice containing punicalagin equal to the MIC value and growth control was incubated with the bacteria final inoculum density of $\sim 10^5$ CFU/mL. The samples were harvested after 1, 2, 3, 6, 9, 12, and 24 hours. The harvested samples (10 μ L) were streaked in brucella agar plate and were incubated at 37°C for 48 hours at anaerobic condition.

Determination of toxin B production

To measure TcdB released into the culture medium, the samples were harvested at 48 hours from MIC plate. An enzyme-linked immunosorbent assay (ELISA) for *C. difficile* toxin A or B (tgcBIOMICS, Bingen am Rhein, Germany) was used according to the manufacturer's instructions to measure TcdB levels.

Statistical analysis

Two-way ANOVA was performed with Dunnett's test using PRISM seven to calculate the antibacterial effect and also

TcdB expression with multiple comparisons. *P*-value <0.001 are considered statistically significant.

Results

Antimicrobial susceptibility

MBC was determined by sub-culturing 10 μ L of samples from each MIC well onto a fresh solid medium (described in the "Materials and methods" section). A number of bacterial colonies were counted. Lowest concentration of pomegranate juice containing punicalagin that killed 100% of bacteria was considered as MBC. The MIC and MBC of pomegranate juice containing punicalagin were found to be 390 μ g/mL for all *C. difficile* hypervirulent strain NAP1/027/BI (results are shown in Figure 1 and Table 1). Growth of *L. lactis* spp., *L. casei*, and *B. animalis* was not inhibited by pomegranate juice containing punicalagin (results are shown in Table 1). Results were obtained in triplicate.

Determination of time period for antimicrobial activity

To determine the time period for antimicrobial activity of the pomegranate juice, an experiment was performed as

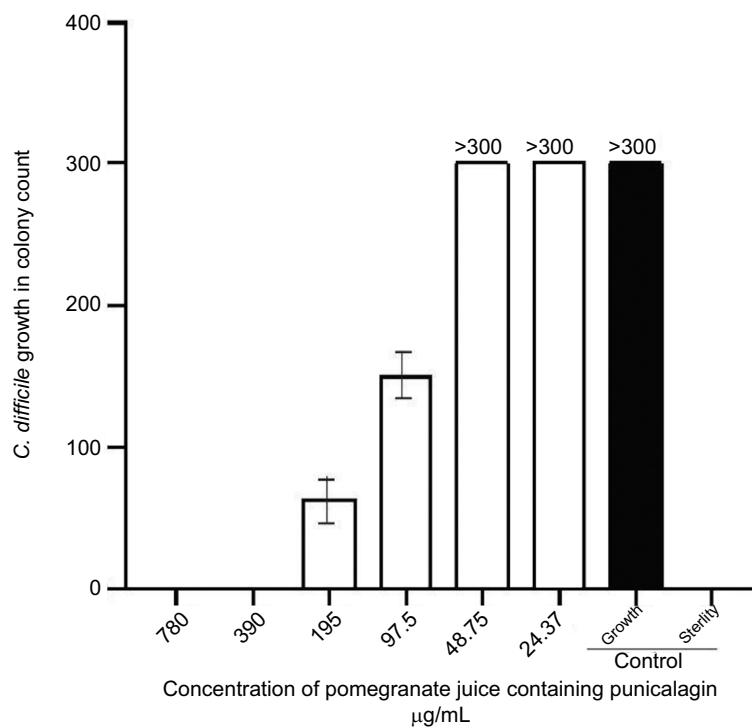


Figure 1 Determination of MBC for *C. difficile* against pomegranate juice.

Notes: Determination of MBC for four different *C. difficile* NAP1/027/BI strains. The final concentrations of pomegranate juice containing punicalagin ranging from 24.37 to 780 μ g/mL were incubated with bacteria. GC and SC were also included. The number of bacteria colonies grown against pomegranate juice were counted. MBC of pomegranate juice containing punicalagin was found to be 390 μ g/mL for all four *C. difficile* strains. Pomegranate juice containing punicalagin inhibited *C. difficile* growth in dose dependent manner.

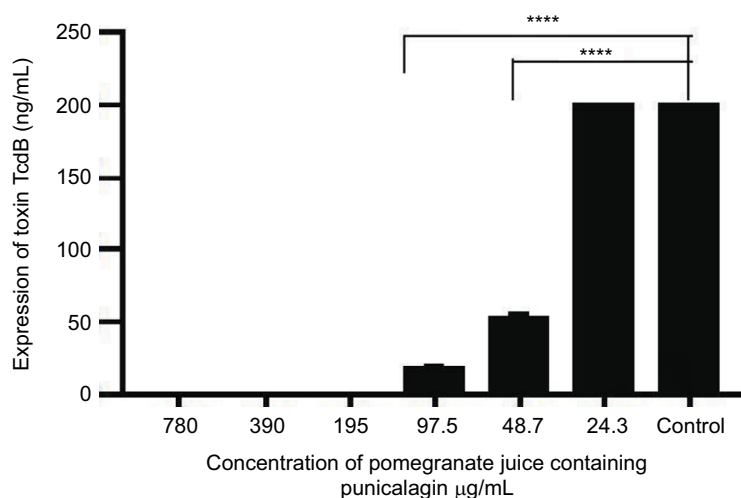
Abbreviations: *C. difficile*, *Clostridium difficile*; GC, growth control; MBC, minimum bactericidal concentration; SC, sterility control.

Table 1 Antimicrobial susceptibility of different bacteria against pomegranate juice

Different bacteria	780 µg/mL	390 µg/mL	195 µg/mL	97.5 µg/mL	48.75 µg/mL	24.37 µg/mL	GC	SC
<i>Clostridium difficile</i> 1	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
<i>Clostridium difficile</i> 2	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
<i>Clostridium difficile</i> 3	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
<i>Clostridium difficile</i> 4	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
<i>Lactococcus lactis</i> spp.	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
<i>Lactobacillus casei</i>	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
<i>Bifidobacterium animalis</i>	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)

Notes: Determination of MIC and MBC for four different *Clostridium difficile* NAP1/027/BI strains, *Lactococcus lactis* spp., *Lactobacillus casei*, and *Bifidobacterium animalis*. The final concentration of pomegranate juice containing punicalagin ranges from 24.37 to 780 µg/mL. GC and SC also included. Culture media having pomegranate juice containing punicalagin at different concentrations and control media were prepared. (+) means growth, (-) means no growth. MIC was identical to MBC.

Abbreviations: GC, growth control; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; SC, sterility control.

**Figure 2** Expression of TcdB in the presence of pomegranate juice.

Notes: Expression of TcdB from *Clostridium difficile* hypervirulent strain in the presence of different concentrations of pomegranate juice containing punicalagin ranging from 24.3 to 780 µg/mL and in the control. ****Indicates toxin expression was greater than 200 ng/mL.

Abbreviation: TcdB, clostridial toxin B.

described in the “Materials and methods” section. *C. difficile* growth found in 1 hour, 2 hour and in growth control plate while all the remaining plates showed no growth. Thus, the time period required for antimicrobial activity of pomegranate juice against *C. difficile* was found to be 3 hours (data not shown).

Determination of TcdB production in the presence of pomegranate juice

To determine the TcdB production of *C. difficile* in the presence of pomegranate juice, an experiment was performed as described in the “Materials and methods” section.

Pomegranate juice containing the concentration of punicalagin ranging from 48.7 to 780 µg/mL decreased the expression of TcdB. Results were obtained in duplicate (results are shown in Figure 2).

Discussion

In this study, pomegranate juice inhibited the growth of *C. difficile* hypervirulent strain NAP1/027/BI but did not inhibit the growth of some probiotics species such as *L. lactis* spp., *L. casei*, and *B. animalis*. A number of other studies have investigated the effects of pomegranate polyphenols on the growth of individual bacterial species. In a recent study, POMx, a pomegranate extract (husks, seeds, and peel remaining after juice production), and pomegranate juice stimulated the growth of bifidobacteria and lactobacilli and inhibited the growth of *B. fragilis* group, *Clostridia*, and Enterobacteriaceae in stool cultures.¹² In another study, POMx stimulated the growth of total bacteria, *Bifidobacterium* spp., and *Lactobacillus* spp. and inhibited only the growth of *Clostridium coccoides*, *Eubacterium rectale* group, and the *C. histolyticum* group.¹³ The growth

of pathogenic *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Clostridia* was significantly inhibited by punicalagins.^{14,15} POMx may be a useful therapy for the prevention of CDI.¹⁶ POMx may inhibit the colonization of pathogen in the colon by inducing the growth of probiotic bacteria in the colon.^{17,18} The probiotic bacteria species such as lactobacilli and bifidobacteria inhibited the growth of pathogenic *C. difficile*.^{19,20} Thus, probiotics may be useful to prevent the colonization of pathogenic species in the lumen and subsequent adhesion and invasion in to the gastrointestinal mucosa. Dietary polyphenols and their metabolites are useful to maintain the gut microbial balance by stimulating the growth of beneficial bacteria and inhibiting the growth of pathogenic bacteria.³¹ Our result suggests that a commercial common pomegranate juice may be useful for maintaining the intestinal microbial balance by stimulating the beneficial bacteria growth, which prevents the colonization of potential pathogens such as *C. difficile* in the gut and also reduce CDI directly by its antimicrobial activity.

In this study, time period required for the antimicrobial activity of pomegranate juice against *C. difficile* was found to be <3 hours. Since *C. difficile* produces toxins and spores during stationary phase, an antimicrobial agent that kills *C. difficile* both in exponential phase and in stationary phase may be predominantly useful in reducing the spore titer and toxin levels in the colon and stool.²¹ In this study, pomegranate juice reducing the TcdB production in *C. difficile* hypervirulent strain NAP1/027/BI may be due to less time period taken to kill *C. difficile*. In contrast, another study showed an increase in toxin TcdA/B production by subinhibitory concentrations of both vancomycin (a cell wall synthesis inhibitor) and metronidazole (a nucleic acid synthesis inhibitor).²²

The drugs of choice for treating CDIs have been antibiotics. However, a few studies have reported strains resistant to metronidazole or with reduced susceptibility to vancomycin.^{4,5} Fidaxomicin has a single target (RNA polymerase), and resistance to fidaxomicin has also already arisen during clinical use.⁶ Lysis of cell wall has been documented in bacteria exposed to phenolic compounds.^{23,24} Disruption of bacterial cell membrane leads to the loss of membrane potential, impaired ATP production, and leakage of intracellular contents.^{25,26} To the best of our knowledge, no reports on antimicrobial resistance to pomegranate polyphenol have been documented, which may be due to its multiple mechanisms of action potentially preventing the formation of resistant strains.

Ideal therapies for CDI would selective target only the specific pathogens and protect the beneficial bacteria in the gut, which is advantageous in the restoration of colonization resistance. Metronidazole and vancomycin currently remain the mainstay antibiotics for the treatment of CDI, but these antibiotics cause significant disruption to the normal gut microbiota.²⁷ Antibiotics treatment repeated for the recurrent CDI promotes dysbiosis, which further disturbs colonization resistance. In addition, repeated use of metronidazole and vancomycin promotes the development of vancomycin-resistant enterococci.²⁸ Recurrence of CDI after treatment with metronidazole and vancomycin was found to be 27.1 and 24.0%, respectively.²⁹ Recurrent CDI mainly results from a disruption of beneficial bacteria in the gut by antibiotic therapy. Pomegranate juice may be useful in maintaining the intestinal microbial balance, and it may be useful to prevent the recurrence of CDI. Pomegranate as well showed a significant anti-inflammatory activity in the gut³⁰ apart from its antibacterial activity against *C. difficile* and prebiotic activity.

Overall, the use of antibiotics to kill toxigenic *C. difficile* has several disadvantages such as resistance development, disruption of normal gut microbiota, recurrence of infection, and enhanced pathogenesis due to increased toxin production. To the best of our knowledge, no reports on microbial resistance to pomegranate polyphenol have been documented and this study showed that pomegranate juice inhibited the *C. difficile* without disturbing the normal microbiota and decreased the toxin production as well. Pomegranate juice is a novel antimicrobial agent that could potentially fulfill the requirements for improved CDI treatment and ability to spare the normal gut microbiota.

Conclusion

Our results suggest pomegranate juice, a common commercial fruit juice, may be a useful antimicrobial agent to prevent or suppress CDI, avoiding the use of antibiotics. Further investigation in a clinical study will be required to determine the potential of pomegranate juice to prevent or suppress CDI.

Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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