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ORIGINAL ARTICLE – OPEN ACCESS

In vitro Activity of Boric Acid Against Biofilm Formation and Biofilm Viability in Medically Important Microorganisms

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ABSTRACT

Objective: Boric acid (BA), a weak acid of boron, is used in many areas and one of the most important boron reserves is located in Türkiye. This study, in addition to investigating how biofilm formation is affected by BA in a dose-dependent manner, aimed to evaluate the minimum inhibitory concentrations (MICs), minimum fungicidal concentrations (MFCs), minimum bactericidal concentration (MBCs) and, anti-biofilm activity of BA against the medically important bacteria and yeast species.

Materials and Methods: In total 19 medically important bacteria and yeast reference strains are chosen where the MICs, MFCs and MBCs values are calculated with broth microdilution method as indicated in Clinical and Laboratory Standards Institute (CLSI) criteria. In addition to the viable microorganisms in biofilm being enumerated, the rates of biofilm inhibition are evaluated with the modified Crystal violet staining method.

Results: MICs values for the bacteria ranged between 61.83–1.93 mg/ml. The lowest MIC value (0.97 mg/ml) and the high rates of biofilm inhibition (93.39%) of fungi strain belong to *C. albicans* ATCC 10231. In addition, it was observed that all dilutions of BA provided significant reductions in viable microorganisms in the biofilm structure.

Conclusion: Antimicrobial and anti-biofilm activity of BA against bacterial and fungal strains with medical importance may indicate that BA may be a promising alternative against medically important pathogens.

Keywords: Boric acid, antimicrobial, anti-biofilm, carbapenem resistance

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INTRODUCTION

Antimicrobial resistance in pathogens is an important problem worldwide increasing morbidity and mortality. Multidrug-resistant microorganisms are difficult to treat and may even be untreatable with traditional antimicrobials. With more than 35,000 people dying for this reason in the same report (1), Centers for Disease Control and Prevention (CDC) declared that about 3 million antibiotic-resistant infections are reported in the USA annually.

Resistance may occur by target site alteration to reduce the drug in the intracellular concentration or inactivation of antibiotics (2), depending on the types of microorganisms and antimicrobials. Besides these mechanisms, biofilm formation may contribute to antibiotic resistance in many microorganisms causing chronic infections. Biofilms are communities of microorganisms surrounded by a self-produced extracellular matrix. By the presence of one or more microbial species, biofilms decrease antimicrobial and disinfectant susceptibility and host defense systems which complicates treatment (3, 4).

The need for new antimicrobial agents particularly for biofilm-forming microorganisms has emerged (5), with the development of new drugs being limited and microorganisms having developed resistance mechanisms against all current antibiotics. Therefore, BA is a weak acid of the boron which can be used in many areas. Its medicinal use comes from its antimicrobial activity and Türkiye besides Russia and the USA is the most important boron deposits (6). BA is known for antibacterial activity and is used as a detergent or antiseptic agent with this property. Also, some studies report the antibacterial activity of BA (7, 8).

In this study, the antibacterial and antifungal activity of BA is tested against standard strains of medically important bacterial and yeast species. Moreover, also tested is the anti-biofilm activity of BA and its effectiveness against the number of viable cells in the biofilm formation. Hence the ability of BA to inhibit biofilm formation, which is another problem in the treatment of infectious diseases, was tested.

MATERIALS and METHODS

This study was approved by Başkent University Institutional Review Board (project no. KA21/382) on 27 September 2021.

Reference Strains. 14 bacterial and 5 yeast standard strains were included in the study; *Staphylococcus aureus* ATCC 6538, *S. aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Acinetobacter baumannii* ATCC 19606, *Klebsiella pneumoniae* ATCC 700603, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus pyogenes* ATCC 19615, *P. aeruginosa* ATCC 15442, *K. pneumoniae* NCTC 13440, *K. pneumoniae* bla_{OXA} confirmed strain (9), *K. pneumoniae* CDC 529, *K. pneumoniae* ATCC 1705, *Candida albicans* ATCC 90028, *C. albicans* ATCC 10231, *Candida krusei* ATCC 6258, *Candida guillermondii* ATCC 6260, and *Candida parapsilosis* ATCC 22019.

Determination of Minimum Inhibition Concentration (MIC)

MIC values were determined by the broth microdilution method according to CLSI criteria (10, 11). Concentrations of BA varied from 123.66 to 0.48 mg/ml. Bacterial dilutions were prepared to 0.5 McFarland turbidity in Mueller–Hinton Broth and diluted 1/100. Yeast suspensions were adjusted to 1 McFarland turbidity in RPMI and diluted 1/100. BA dilutions and microorganisms were added 1:1 in 96-well microplates. After the incubation period, MIC values were determined by reading the microplates in the spectrophotometer at 550 nm (BioTek Instruments, ELX 800, USA).

Determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

In the microplate in which MIC was studied, 10 µl of each dilution was taken and inoculated on Tryptic Soy Agar for bacteria and Sabouraud Dextrose Agar for yeasts. At the end of the 24-hour incubation, MBC and MFC were defined (10, 11). Therefore, MBC and MFC values were the lowest concentrations required to kill bacteria and fungi, respectively, after the incubation period.

Biofilm Formation Assay

The method described by Stepanović et al. (12) with slight modification was applied in triplicate (13). Biofilm formation was evaluated as *ODc* = mean *OD* of negative control + 3 × *SD* of negative control (12). *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 6538, known as biofilm-forming isolates, were used as a positive control, while *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213, known as non-biofilm-forming isolates, were used as negative controls, where, following biofilm formation, Crystal violet staining procedure was applied and optical density (OD) was measured at 550 nm (BioTek Instruments, ELX 800, USA).

Evaluation of Anti-biofilm Activity of BA

In 96-well flat-bottom microplates, the anti-biofilm activity of BA was determined on reference strains in a dose-dependent manner. Microorganisms were prepared similarly to the procedure used for MIC calculation. With a 2-fold serial dilution, different concentrations of BA were obtained and tested. BA dilution and microorganism suspension were added suitably 1:1. After 24 hours of incubation, microplates were stained with the modified Crystal violet staining method and read in the spectrophotometer (13). The rates were calculated as follows: "Control OD- Sample OD]/ Control OD) × 100" (14).

Number of Viable Cells After BA Treatment

Biofilm was formed by reference strains in 96-well flat-bottom microplates and incubated at 24 hours. Before the microplates, which were kept during the incubation period, were sonicated 30% amplitude for 10 seconds at the end of the period, BA suspensions with different concentrations (123.66–0.48 mg/ml) were added to the biofilm-formed wells up to 200 µl. After sonication, 100 µl of each well was inoculated into the media. Colonies were counted after 24 hours of incubation (15). The procedure was applied in triplicate.

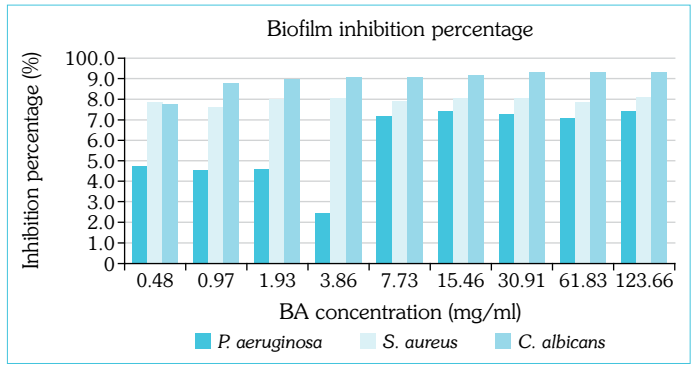

Figure 1. Percentage of anti-adhesion activity of boric acid on reference strains

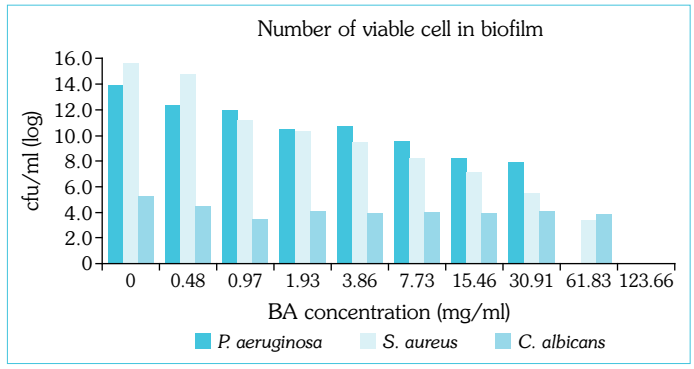

Figure 2. Number of viable cells in biofilms before and after the exposure of different BA concentrations

Statistical Analysis

IBM SPSS Statistics version 25.0 was used for the statistical analysis. Fitted by the full factorial two-way ANOVA model were the interaction of the model established in the form of microorganism groups, BA concentrations, measurement replications (3 replications) and the interaction of microorganism group and BA concentrations on the number of microorganisms. Response variable were log-scaled. Post-Hoc comparisons were performed using Türkiye HSD test. The statistical level was sat at $p<0.05$.

RESULTS

Antibacterial and Antifungal Effects of BA

MIC, MBC/MFC values were calculated by CLSI criteria. The lowest MIC values were 1.93 mg/ml for tested bacteria and 0.97 mg/ml for fungi. While MBC values differed from >123.66 to 15.46 mg/ml for bacteria, it varied from 1.93 to 61.83 mg/ml for fungi. The lowest MBC value was obtained from *S. pyogenes* ATCC 19615 reference strains, 15.46 mg/ml (Table 1, 2).

Table 1. MIC and MBC values of BA for bacterial strains

Microorganisms	Properties	MIC (mg/ml)	MBC (mg/ml)
S. aureus ATCC 29213	MSSA	15.46	123.66
K. pneumoniae ATCC 700603	ESBL-producing isolate	15.46	123.66
P. aeruginosa ATCC 27853	Quality control strain	7.73	>123.66
S. epidermidis ATCC 12228	Quality control strain	7.73	123.66
E. coli ATCC 25922	Quality control strain	7.73	123.66
E. faecalis ATCC 29212	Quality control strain	1.93	3.86
S. aureus ATCC 6538	MRSA-Biofilm-producing isolate	3.86	123.66
P. aeruginosa ATCC 15442	Biofilm-producing isolate	7.73	123.66
S. pyogenes ATCC 19615	Quality control strain	3.86	15.46
K. pneumoniae NCTC 13440	bla_{VIM} carrying isolate	30.91	123.66
K. pneumoniae bla_{OXA48}	bla_{oxa48} carrying isolate*	61.83	123.66
K. pneumoniae CDC 529	bla_{NDM} carrying isolate	30.91	123.66
K. pneumoniae ATCC 1705	bla_{KPC} carrying isolate	30.91	123.66
A. baumannii ATCC 19606	Biofilm-producing isolate	15.46	123.66

*: This strain carrying bla_{OXA48} was previously confirmed by Üsküdar Güçlü et al. (9).

Table 2. MIC and MFC values of BA for fungal strains

Microorganisms	MIC (mg/ml)	MFC (mg/ml)
C. guillermondii ATCC 6260	7.73	30.91
C. krusei ATCC 6258	0.97	7.73
C. albicans ATCC 10231	0.97	61.83
C. albicans ATCC 90028	1.93	61.83
C. parapsilosis ATCC 22019	1.93	1.93

MIC: Minimum inhibition concentration; MFC: Minimum fungicidal concentration; BA: Boric acid.

Anti-biofilm Activity of BA

Anti-biofilm activity of BA was determined in a dose-dependent manner. In all tested BA concentrations, ranging from 0.48 to 123.66 mg/ml, biofilm formation was inhibited in all tested isolates. While biofilm inhibition percentage for *C. albicans* ATCC 10231 ranged from 93.4 to 77.8%, for *S. aureus* ATCC 6538, it varied between 80.7 to 76.4%, and, in *C. albicans* ATCC 10231 and *S. aureus* ATCC 6538, the biofilm formations were inhibited significantly even at the lowest concentration of BA. The lowest percentage of biofilm inhibition for *P. aeruginosa* ATCC 15442 was obtained at BA concentration 3.86 mg/ml, 24.4% (Fig. 1).

Number of Viable Cells in The Biofilm Structure After BA Exposure

The number of viable cells in the biofilm were quantified for *P. aeruginosa* ATCC 15442, *S. aureus* ATCC 6538 and *C. albicans* ATCC 10231. The number of viable microorganisms treated with different concentrations of BA ranging from 0.48 to 123.66 mg/ml was compared with the number of viable microorganisms without BA treatment (Fig. 2). At the highest concentration of BA, there were no viable bacteria and fungi in the biofilm (100% of inhibition). The change in the number of microorganisms according to the different BA concentrations tested for each microorganism was found to be statistically significant ($p<0.001$). For each microorganism, a statistically significant difference was found between the number of microorganisms in the group without BA (cfu/ml) and the number of microorganisms in 0.48 mg/ml BA and other BA concentrations ($p<0.001$) (Table 3). As seen in Figure 2, the number of microorganisms at 0.97–123.66 mg/ml BA concentrations decreased as the BA concentration increased, but the change in the number of microorganisms between these concentrations was not statistically significant ($p>0.05$).

DISCUSSION

BA is a weak acid of boron with significant reserves in many countries of the world. The most important boron resources are located in Türkiye (6). The antimicrobial activity of this chemical substance is also very important in many fields. There are limited studies on the anti-biofilm activity of BA on medically important bacterial and fungal strains (16), although some studies have revealed its antimicrobial activity against certain microorganisms. Therefore, it was aimed to evaluate the antimicrobial and anti-biofilm activity of BA on medically important yeast and bacterial pathogens.

Determining MIC, MBC and MFC values of BA is significant for evaluating its efficacy on bacteria and fungi (17). In this current study, MIC, MBC and MFC of medically important pathogens were determined (Table 1, 2). Among these strains, MICs were determined as 3.86, 15.46, 7.73, 15.46 and 7.73 mg/ml for methicillin-resistant *S. aureus* (MRSA) ATCC 6538, ESBL positive *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853, *A. baumannii* ATCC 19606 and biofilm positive *P. aeruginosa* ATCC 15442, respectively. MBCs were calculated as 123.66 mg/ml for bacteria (except *P. aeruginosa* ATCC 27853 and *S. pyogenes* ATCC 19615) (Table 1). MICs varied between 1.93 to 61.83 mg/ml. According to a study, while the MBC value was found as 7.60 mg/ml (8), the MIC value for *P. aeruginosa* ATCC 27853 was calculated as 7.60 mg/ml. Even though in the current study the MIC value for

Table 3. Statistical analysis of number of viable cell in biofilm

BA Concentration (mg/mL)	Species		
	P. aeruginosa	***S. aureus***	***C. albicans***
Without BA			
0.48	0.0E+0±0.0E+0	0.0E+0±0.0E+0	0.0E+0±0.0E+0
0.97	2.6E+06±7.5E+07^{Aab}	5.6E+02±2.4E+03^{Bc}	8.7E+02±7.5E+03^{Cab}
1.93	6.1E+06±1.6E+08^{Aab}	4.4E+04±3.2E+05^{Bc}	2.6E+03±1.2E+04^{Cab}
3.86	1.0E+09±3.0E+09^{Aab}	2.6E+06±1.5E+07^{Ac}	2.6E+03±9.0E+03^{Aab}
7.73	5.2E+09±5.3E+10^{Aab}	2.8E+07±1.8E+08^{Bc}	2.0E+03±1.04E+04^{Bab}
15.46	4.4E+09±3.0E+10^{Aab}	6.2E+08±2.5E+09^{Ac}	2.6E+03±9.0E+03^{Bab}
30.91	8.7E+10±8.0E+11^{Aab}	1.7E+09±2.1E+10^{Bc}	3.0E+03±1.2E+04^{Cab}
61.83	3.6E+11±2.1E+12^{Aab}	3.0E+10±1.7E+11^{Aab}	1.0E+03±3.0E+03^{Aab}
123.66	2.6E+14±5.0E+15^{Aab}	1.7E+14±6.0E+14^{Ac}	2.6E+03±3.0E+04^{Aab}
Significances (p-values)**	Species	<0.001	
	BA concentrations	<0.001	
	Interaction effect	<0.001	

* Values were summarized with the means and standard deviations. Summary statistics were based on the untransformed values; **: A full factorial two-way ANOVA model were fitted. Response variable were log-scaled. The experiment is replicated 3 times at each factor combination of Species and BA Concentrations. Post-Hoc comparisons were performed using Türkiye HSD test. Groups not having letters in common were found to be statistically significantly different. Capital and small letter corresponds to the Pos-Hoc comparison between Species and BA Concentration groups, respectively

P. aeruginosa ATCC 27853 was in concordance with the previous study, 7.73 mg/ml, Sayın et al. (7), demonstrated a significantly lower MIC value for the same reference strain, as 0.385 mg/ml.

Carbapenemase-producing *Enterobacterales* have emerged worldwide and have been associated with numerous epidemics. Treatment methods against carbapenemase-producing *Enterobacterales* are limited and there is a need for new antimicrobial agents for infections caused by these strains (18). In this study, BA activity was tested on *K. pneumoniae* strains carrying the most frequently encountered carbapenemase resistance genes, bla_{KPC}, bla_{NDM}, bla_{VIM} and bla_{OXA48}. MIC values of BA were 30.91, 61.83, 30.91 and 30.91 mg/ml for *K. pneumoniae* carrying bla_{VIM}, bla_{OXA48}, bla_{NDM} and bla_{KPC}, respectively. MBC values were calculated as 123.66 mg/ml for each of these four strains. As far as we know, this study is the first study that evaluates BA activity against *K. pneumoniae* isolates carrying carbapenem-resistant genes.

Candida species have the ability to cause opportunistic infections. Antifungal resistance is a growing problem and according to CDC data during 2013–2017, the average incidence of candidemia was approximately 9 per 100,000 people, and approximately 7% of all *Candida* blood samples were resistant to the antifungal drug fluconazole (19). The antifungal activity of BA has been the subject of some studies (20). It can even be used sporadically in the treatment of vaginal candidiasis infections. In a study conducted by Gavilanes-Martínez et al. (21), 5% BA suspension demonstrated the highest antifungal activity. In addition, several retrospective studies showed that 300–600 mg of BA for the treatment of vaginal candidiasis was applied and the mycological cure rate varied between 40%–100% (22, 23). In this current study, while MBC varied ranging 1.93–61.83 mg/ml (Table 2), MIC of Candida spp. was significantly low, 0.97–7.73 mg/ml.

The ability of microorganisms to form biofilm is another important limitation for the treatment of infections. The biofilm inhibition percentages of BA in a dose-dependent manner and the number of viable cells in biofilm after BA treatment were evaluated. Even at the lowest concentration of BA (0.48 mg/ml), where in *P. aeruginosa*, the lowest biofilm inhibition percentage was observed at 3.38 mg/ml, where 7.73 mg/ml was the MIC value, the biofilm inhibition percentages of *C. albicans* and *S. aureus* strains were 77.78% and 78.83%, respectively.

The effects of BA on the number of viable microorganisms in biofilm were tested by the colony counting method. With it being stated that 4% BA suspension was a strong biofilm inhibitor (24), a previous study evaluated the anti-biofilm activity of BA on MRSA and *P. aeruginosa* resistant to quinolone. In the current study, it was found that BA reduced the number of viable microorganisms in biofilm significantly even for MRSA, *P. aeruginosa* and *C. albicans*. No viable microorganisms were detected in all three isolates at the highest concentration of BA. Even in the lowest concentration, BA was able to reduce the number of viable microorganisms in biofilm in all tested isolates (Fig. 2). The number of viable bacteria in biofilm was accounted for approximately 10^{15} and 10^{12} CFU/ml. When 15.46 mg/ml BA was applied, the number of viable microorganisms decreased to 10^{8}, 10^{7} and 10^{3} CFU/ml for *P. aeruginosa*, *S. aureus* and *C. albicans*, respectively. The statistical analysis obtained from the colony counting method demonstrated that concentrations of 7.73 mg/ml and above of BA provided a statistically significant reduction in the number of viable microorganisms in the biofilm (p=0.043). The data obtained from this current study provide significant knowledge for the activity of BA against biofilm formation and biofilm viability, as the biofilm formation inhibition and BA efficacy on viable microorganisms was never evaluated in previous studies.

CONCLUSION

In this study, the antimicrobial and anti-biofilm activity of BA on medically important bacterial and fungal strains was tested. Multi-drug-resistant isolates carrying specific antibiotic resistance genes, such as carbapenem-resistant Enterobacterales, have become an important healthcare problem with limited treatment options. Therefore, BA activity against these isolates is gaining importance in the development of new antimicrobial agents. Although the toxic dose of BA in humans varies according to the route of administration, where even in biofilm, the efficacy of BA on the number of viable microorganisms is promising, and biofilm formation can be inhibited up to 80%–90%. Therefore, these data can provide significant knowledge for further studies.

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