Effects of boric acid and potassium metaborate on cytokine levels and redox stress parameters in a wound model infected with methicillin-resistant *Staphylococcus aureus*

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Abstract. Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are usually found in hospital settings and, frequently, in patients with open wounds. One of the most critical virulence factors affecting the severity and recurrence of infections is the biofilm; increasing antibiotic resistance due to biofilm formation has led to the search for alternative compounds to antibiotics. The present study aimed to use boric acid and potassium metaborate against MRSA infection in a fibroblast wound model. For this purpose, a two-part experiment was designed: First, MRSA strains were used for the test, and both boric acid and potassium metaborate were prepared in microdilution. In the second step, an MRSA wound model was prepared using a fibroblast culture, and treatments with boric acid and potassium metaborate were applied for 24 h. For the evaluation of the effects of treatment, cell viability assay (MTT assay), analysis of redox stress parameters, including total oxidant status and total antioxidant capacity analyses, lactate dehydrogenase analysis and immunohistochemical staining were performed. In addition, IL-1β and IL-10 gene expression levels were assayed. According to the results, potassium metaborate was more effective and exhibited a lower toxicity to fibroblast cells compared to boric acid; moreover, potassium metaborate decreased the level of prooxidant species and increased the antioxidant status more effectively than boric acid. The IL-1β level in the bacteria group was high; however, boric acid and potassium metaborate significantly decreased the expression levels of inflammatory markers, exhibiting the potential to improve the resolution of the lesion. On the whole, the findings of the present study suggest that boric acid and potassium metaborate may be effective on the tested microorganisms.

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Introduction

According to international literature data, a wound is defined as a condition that disrupts the integrity of the membranes (skin or mucosa), a physical trauma induced by factors, such as cuts, abrasions or burns. Wounds that tend to become chronic (microorganism origin) are a concern in developed countries, as they are associated with high costs and a negative impact on the quality of life of affected individuals. In particular, biofilm formation is considered one of the most critical factors in the chronicity of wounds (1). By forming a biofilm (polysaccharide-based), microorganisms form a polymicrobial complex structure that allows attachment to living and non-living surfaces, and thus adhere to the wound

surface (2). It has been determined that bacteria able to develop a biofilm structure are more resistant to antibiotics (3,4). *Staphylococcus aureus*, which is responsible for the majority of nosocomial infections, is a cocci-like non-encapsulated bacterium under a Gram-positive coloration. As demonstrated below, the antibiotic susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) was determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (5). MRSA was sensitive to amikacin (30 μg/ml), fusidic acid (10 μg/ml), gentamicin (10 μg/ml), levofloxacin (5 μg/ml), linezolid (10 μg/ml), penicillin (1 μg/ml), trimethoprim sulfamethoxazole (1.25+23.75 μg/ml) and it exhibited resistance to ciprofloxacin (5 μg/ml) (intermediate), erythromycin (30 μg/ml), clindamycin (2 μg/ml) and tetracycline (30 μg/ml).

Methicillin is an effective antibiotic used in the treatment of *Staphylococcus aureus*; however, some strains are methicillin-resistant (MRSA). MRSA infections are usually observed in open wounds in hospitalized patients (6). Chronic biofilm-associated infections caused by *Staphylococcus aureus* often lead to a significant increase in morbidity and mortality rates, particularly when associated with permanent medical devices (6,7). Biofilm plays a role in the adhesion of microorganisms to a surface. It is a layer of extracellular polymeric structures formed by the microorganism, environmental organic and inorganic substances and proteins. The biofilm formed by microorganisms protects them from phagocytosis and antibiotics. Therefore, it leads to sepsis and chronic infections. The icaA and icaD genes are important in the biofilm layer in the structure of *Staphylococcus aureus*. One of the proteins produced by the icaA and icaD genes, the 'biofilm-associated protein' is one of the necessary structures for the biofilm formation of *Staphylococcus aureus* (7). In addition, the presence of biofilms on chronic wounds inducing community-acquired *Staphylococcus aureus* has been reported as 78.2% in wound studies (8). Chronic wound biofilms represent a significant treatment challenge by demonstrating recalcitrance towards antimicrobial agents. In addition, the delayed wound healing process caused by biofilm is one of the main reasons. *Staphylococcus aureus* and its associated biofilm are among the dominant causes of persistent infections in chronic wounds. After MRSA attaches to cells, the biofilm develops into a 'mat' composed of an extracellular DNA (eDNA) and proteinaceous matrix. After reaching confluency, a period of cell mass egress occurs, in which a subpopulation of cells is released from the biofilm via nuclease-mediated eDNA degradation to allow the formation of three-dimensional microcolonies consisting of different foci of cells that remain attached during the exit phase. This stage is characterized by rapid cell division, forming robust aggregates of proteins, including phenol-soluble modulins and eDNA (9).

Therefore, in recent years, the increasing antibiotic resistance, the increase in biofilm formation and recurrent wounds cases have led to the search for alternative compounds to stimulate/improve the outcomes of antibiotic treatment. Recently, various antibacterial studies with boron and boron compounds have attracted attention (10-14).

The ability of boron to form covalent bonds with carbon renders it very useful in the drug discovery process. Trivalent boron derivatives have a trigonal planar structure and are electrophiles. By accepting a pair of electrons, they convert in a tetrahedral configuration and become nucleophile agents. This transformation explains the capacity of various boron derivatives to inhibit a large array of enzymes (15,16). Bortezomib is a boronic acid derivative approved by the FDA as an anticancer treatment, acting as a proteasome inhibitor (17). An alkaline type of boron, potassium metaborate, which is one of the most critical boron compounds, is a solvent for casein and an important additive in the fabrication processes for stainless steel and other non-ferrous metals (18).

Boron compounds are used effectively especially in vaginal yeast infections caused by dimorphic fungus *Candida albicans* (19). It has been reported that the application of 3% boric acid to deep wounds significantly contributes to the healing cascade and the reduction of hospital duration in intensive care units (20,21). Sodium pentaborate has been proven to increase collagen deposition and favor wound contraction *in vitro*, as well as to increase cell migration, superoxide dismutase activity and the expression levels of gene associated with vital wound healing (22). The same formulation has been found to increase the healing of burn wounds by increasing capillary density and fibroblastic activity (23,24). In light of all this information, the present study aimed to examine the effects of boric acid and potassium metaborate on the healing process of MRSA-derived wounds, using a cell culture method. For the evaluation of the efficacy of the test compounds, redox stress parameters, as well as inflammation ones were employed.

Materials and methods

MRSA strain. Community-acquired MRSA bacteria were isolated from a chronic non-healing wound sample which was sent to the Medical Microbiology Laboratory of Ataturk University (Erzurum, Turkey). The VITEK 2 system uses a fluorogenic methodology for organism identification and a turbidimetric method for susceptibility testing. MRSA bacteria isolated from a patient sample, for which ethical approval was obtained from the Ataturk University Faculty of Medicine Clinical Research Ethics Committee (decision date, 04.11.2021; decision no. 23; and meeting no. 07) was used in the study. All necessary consents and permissions were obtained for the patient samples used in the study. The ethics committee document obtained from the authors' institution includes all consent forms. The wound sample sent to the clinical microbiology laboratory was evaluated as a community-acquired agent. The causes of this (culture positivity outside the hospital or within the first 48 h of hospitalization, no previous MRSA infection or colonization, hospitalization over the past year, residing in a nursing home, no surgery and indwelling catheter or crossing the skin, or the absence of a medical device) were taken into account. Bacteria were selected according to these criteria.

Reagents and chemicals. Boric acid, potassium metaborate, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), fetal calf serum (FCS), antibiotic antimitotic solution (x100), L glutamine, trypsin-EDTA, paraformaldehyde and ethanol were obtained from MilliporeSigma.

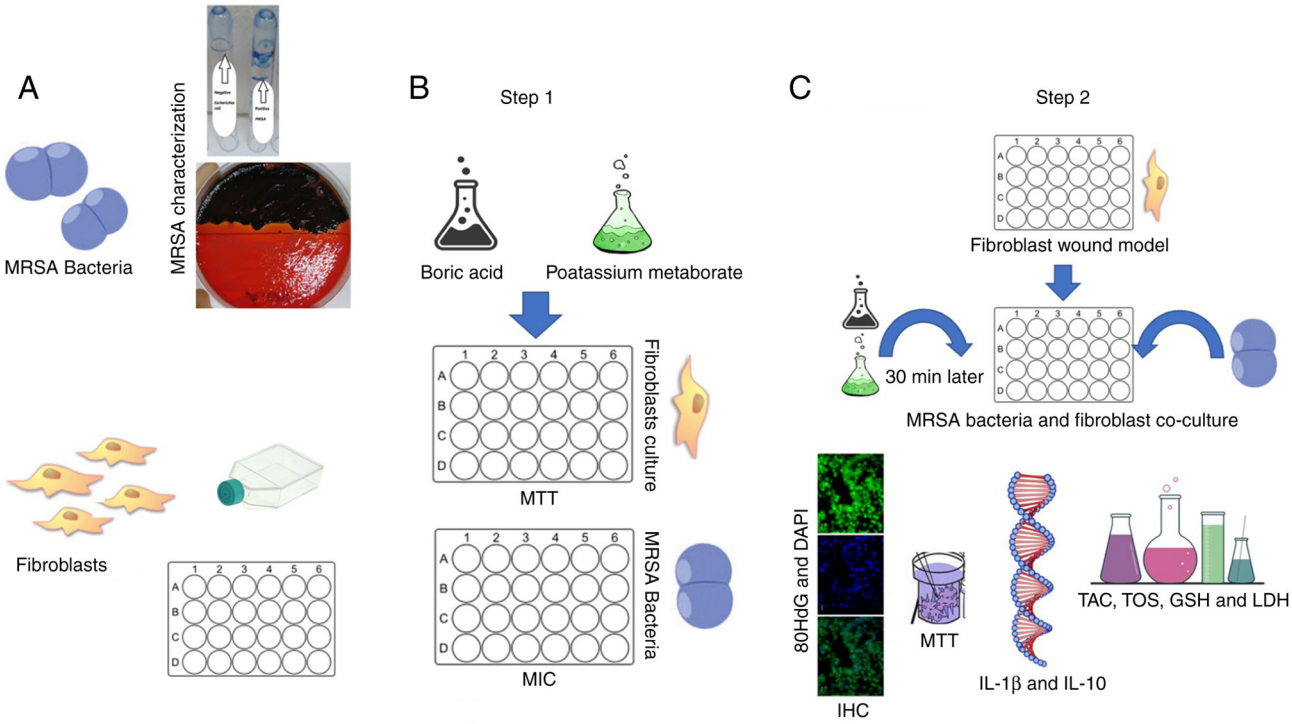

Figure 1. Experimental design. (A) MRSA bacterial characterization and fibroblast culture was made; (B) Step 1: Boric acid (1,250 μg/ml) and potassium metaborate (600 μg/ml) toxicity tests for concentration and MRSA bacteria MIC were assessed. (C) Step 2: Wound model was prepared than bacteria used for co-culture and after 30 min the treatment were added; 24 h later, MTT assay, TOS and TAC, LDH, Gr, immunofluorescence, and IL-1β and IL-10 expression analyses were performed. MRSA, methicillin-resistant *Staphylococcus aureus*; MIC, minimal inhibitory concentration; TOS, total oxidant status; TAC, total antioxidant capacity; LDH, lactate dehydrogenase; GSH, glutathione.

Experimental design. The experiment was designed in two different parts: The first part consisted of the evaluation of the boric acid (39, 78, 156, 312, 625, 1,250, 5,000 and 10,000 μg/ml) and potassium metaborate (9.4, 18.75, 37.5, 75, 150, 300, 600, 1,200 and 2,400 μg/ml) minimal inhibitory concentration (MIC) and cell (fibroblast) culture toxicity. The antibacterial effects of boric acid and potassium metaborate were analyzed according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) (25). Community-acquired MRSA bacteria were isolated from a chronic non-healing wound sample sent to the Medical Microbiology Laboratory of Ataturk University (as aforementioned). MRSA strains were used for the test. In microbiology, the MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism following overnight incubation. MICs were defined as the lowest concentration of boric acid and potassium metaborate inhibiting the visible growth of the bacteria. The MICs of boric acid and potassium metaborate were conventionally determined in triplicate, for each bacterial strain by the microdilution broth method as described by the NCCLS (25). Serial dilutions of both boric acid and potassium metaborate were prepared in microdilution with concentrations ranging between 10 mg/ml (40 mM) and 0.02 mg/ml (40 mM). 0.62 mg/ml potassium metaborate and, 2.5 mg/ml boric acid showed minimal inhibition on MRSA.

In the second step, a Methicillin resistant *Staphylococcus aureus* wound model was induced, using a fibroblast cell line; boric acid (1,250 μg/ml) and potassium metaborate (600 μg/ml) were applied for 24 h treatments. These concentrations were preferred as these did not exhibit toxicity at these ranges. After the end of experiment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as well as total antioxidant capacity (TAC), total oxidant status (TOS), glutathione reductase (Gr), lactate dehydrogenase (LDH) and immunohistochemical analyses were performed (Fig. 1).

Cells and cell cultures. For the present study, fibroblast cell (L929, CRL-6364, ATCC) cultures were obtained from the Department of Medical Pharmacology of Ataturk University (Erzurum, Turkey).

Briefly, the cells were re-suspended in fresh medium (DMEM), fetal bovine serum (FBS) 10% and antibiotic 1% (penicillin, streptomycin and amphotericin B) (MilliporeSigma). The cells were then seeded in 24 well plates (Corning, Inc.) and stored in an incubator (5% CO_2; 37˚C), as previously described (21). After obtaining 85% confluency, the wound model was created using 100-μl yellow pipet tip. According to the McFarland 0.5 scale, the bacterial suspension was then added to the cell culture. After 30 min, treatment with boric acid (1,250 μg/ml) and potassium metaborate (600 μg/ml) was applied for 24 h.

MTT assay. At the end of the two parts of the experiment (following 24 h of treatment with boric acid and potassium metaborate), 10 μl MTT solution (MilliporeSigma) were added to each well plate and the samples were incubated for 4 h; 100 μl DMSO (MilliporeSigma) was incorporated into all

wells to dissolve the formazan crystals. The optical density of the solutions was read at 570 nm using a Multiskan™ GO microplate spectrophotometer (23).

The extraction buffer was considered as a blank and the optical density in the control group (untreated cells) was considered as 100% viability. The relative cell viability (%) was calculated as (A570 of treated samples/A570 of untreated samples) x100.

Evaluation of TOS and TAC. TOS and TAC (Rel Assay Diagnostics) evaluations were performed on cell supernatants according to the manufacturer's instructions (Multiskan™ GO microplate spectrophotometer).

For the TOS assay, the oxidants from the biological sample oxidize the ferrous ion-chelator complex to ferric ion; the ferric ions form a complex with a chromogen and the color intensity is evaluated spectrophotometrically (530 nm), with calibration being performed using hydrogen peroxide (Rel Assay Diagnostics).

In the TAS assay, antioxidants in the cell medium reduce the colored 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (radical to colorless reduced ABTS form; the dynamics of absorbance at 660 nm are associated with the total antioxidant level in the sample). For calibration of the assessment, a stable antioxidant standard solution (Trolox) (Rel Assay Diagnostics) was used.

Gr and LDH assay. Glutathione plays a key role in oxidative stress and Gr is responsible for maintaining the supply of reduced glutathione. On the other hand, LDH plays a critical role in the cell energy process and LDH assay is generally used to screen for tissue damage. Supernatants stored at -80˚C were used for the evaluation of LDH (Elabscience), as well as Gr activities, using commercial ELISA kits, according to the manufacturer indications (cat. no. E-EL-R0026, Elabscience) The absorbance was measured using a reader (Multiskan™ GO microplate spectrophotometer).

MRSA identification. Community-acquired MRSA bacteria were isolated from a chronic non-healing wound sample which was sent to the Ataturk University Medical Microbiology Laboratory and included in the study. The sample was inoculated on 5% sheep blood, McConkey agar and mannitol salt agar (MSA) (MilliporeSigma) media and incubated at 37˚C for 24-48 h. The transplanted plates were examined using the Gram staining (MilliporeSigma) method as follows: i) Applying a primary stain (crystal violet) (MilliporeSigma) to a heat-fixed smear of a bacterial culture (1 min, 25˚C); ii) the addition of iodine, which binds to crystal violet and traps it in the cell (1 min, 25˚C); iii) rapid decolorization with ethanol or acetone (10 sec, 25˚C); iv) counterstaining with safranin (MilliporeSigma) (1 min at room temperature) (26) at the end of the 48-h time point.

The distinction between the causative microorganism and the contaminant of the growth was decided according to the results of direct microscopic examination. At the same time, Gram-stained preparations were prepared from the wound sample; x100 objective microorganism morphology was evaluated under a light microscope (Leica DM300; Leica Microsystems GmbH). The MRSA strain was detected by appropriate conventional tests.

Determination of microorganism antibacterial susceptibility. The automated system VITEK 2 (bioMérieux) was used. The VITEK 2 system uses a fluorogenic methodology for organism identification and a turbidimetric method for susceptibility testing. Available test kits include Gram-negative Bacillus identification, Gram-positive cocci identification, Gram-negative susceptibility and Gram-positive susceptibility; sensitivities were interpreted according to EUCAST criteria (5).

Kirby-Bauer disk diffusion method. MRSA colonies, which were cultured for 24 h, were inoculated into Mueller-Hinton medium (MilliporeSigma) with a swab after adjusting the appropriate turbidity in sterile saline with the McFarland 0.5 chart. Subsequently, Sterile Paper discs (6 mm in diameter; Oxoid Antibacterial Susceptibility Blank Test Disc, Oxoid Limited) were impregnated with 20 μg/ml and placed in Mueller-Hinton medium. Zone diameters were measured following 24 h and 37˚C of incubation.

Determination of the MIC. The antibacterial effects of boric acid and potassium metaborate were analyzed according to the recommendations of the NCCLS (25). MRSA strains were used for the test.

The MICs of boric acid and potassium metaborate were conventionally determined in triplicate for each strain by the microdilution broth method as described by the NCCLS (25). Serial dilutions of both boric acid and potassium metaborate were prepared in microdilution with concentrations ranging between 10 mg/ml (40 mM) and 0.02 mg/ml (40 mM). These concentration ranges were determined by experimentation and by Yılmaz (27). Due to the lack of sufficient resources in the literature, the appropriate concentration range was determined as done in the present study. The concentration ranges have been previously described by Yılmaz (27). MRSA colonies prepared according to the McFarland 0.5 scale with serial dilutions were inoculated to all wells as 100 μl. Subsequently, 100 μl tryptic soy broth (TSB) (MilliporeSigma) medium and a solution of boric acid and potassium metaborate were added to the wells by dilution. The last two wells consisted of the positive control containing the microorganism and medium, and the negative control containing the medium and boron derivatives. The samples were incubated for 24 h at 37˚C. MIC values were determined depending on the formation of agglutination.

Biofilm evaluation using the microplate method. The MRSA strain was incubated in TSB medium at 37˚C for 18-24 h. Bacterial suspensions were prepared by standardizing them according to the McFarland 0.5 chart; 100 μl were added to the flat-bottomed wells and incubated at 37˚C for 24 h. At the end of the incubation period, the wells were washed with distilled water and the cell residues associated with the biofilm were stained with 1% crystal violet (MilliporeSigma) for 37˚C for 15 min. Biofilms observed in bacteria were photographed after the excess dye was washed off with water. To quantitatively determine biofilm formation, optical densities were measured on an ELISA reader (Biotek ELX800; BioTek Instruments, Inc.) at OD 450 - OD 630 nm. During the test, sterile TSB was used as a negative control. The *E. coli* ATCC 25922 bacterial strain obtained from Ataturk University Medical Faculty

Medical Microbiology Laboratory was used as a negative control.

Biofilm evaluation using the tube method. A single colony was taken from MRSA pure cultures, inoculated into 5 ml TSB medium containing 0.25% glucose, and incubated at 37˚C for 24 h. Subsequently, the tube contents were emptied and kept at room temperature for 30 min with 1% saffron (MilliporeSigma) solution. At the end of this period, the dye was poured, and the tubes were washed twice with sterile PBS. The tubes were inverted and allowed to dry on blotting paper. The following day, the evaluation was performed (+), (++), (+++) and (-) according to the formation and density of a colored film layer on the inner surface of the tube. The *E. coli* bacterial strain was used as a negative control. All tests were performed as three repetitions, as previously described (22).

Biofilm evaluation using the Congo red agar method. Congo red medium was prepared to contain 50 g sucrose, 37 g brain heart infusion (BHI) agar (MilliporeSigma) and 0.8 g Congo red (MilliporeSigma) per liter. Following inoculation on MRSA Congo red agar, incubation was performed at 37˚C for 24 h. As a result of incubation, the strains forming dry crystallized black colonies were evaluated as biofilm-positive, and the strains forming red or pink colonies were evaluated as biofilm-negative. The *E. coli* bacterial strain was used as a negative control. This experiment was studied in two repeats; three methods are used to determine the presence of *in vitro* biofilm in the microbiological analysis. The presence of biofilm was detected using *in vitro* methods, as previously described (22).

RNA isolation and cDNA synthesis. The RNA of cells was isolated using the High Pure RNA Isolation kit (cat. no. 11828665001, E.Z.N.A.®; Roche Diagnostics). The RNA concentration was measured using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc.). RNA samples for cDNA were performed using the Transcriptor First Strand cDNA Synthesis kit (cat. no. 04896866001, Roche Diagnostics) according to the specified protocol (28).

Quantitative PCR (qPCR). qPCR was performed using primers constructed for IL-1β, IL-10 and β-actin. Briefly, 1 μl Primer Probe mix, 3 μl of cDNA, 3 μl of Fast Start Essential DNA Probes Master mix (Roche Diagnostics) and 13.25 μl distilled water were added to each tube to be used (final volume is 20 μl). The PCR cycling conditions were as follows: 5 min at 95˚C, 10 sec at 95˚C and 30 sec at 60˚C, covering a period of 45 cycles. The results were calculated by calculating the $2^{-\Delta\Delta Cq}$ values (22). The primers used were as follows: β-actin forward, 5'-ATGGATGACGATATCGCTGCG-3' and reverse, 5'-CTAGAAGCACTTGCGGTGCA-3'; IL-1β forward, 5'-ATGGCAACTGTTCCTGAACT-3' and reverse, 5'-TTAGGAAGACACAGATTCCAT-3'; and IL-10 forward, 5'-ATGCCTGGCTCAGCACTGC-3' and reverse, 5'-TTAGCTTTTCATTTTGATCATCA-3'.

Immunofluorescence analysis. The cells cultivated in cell culture were incubated at room temperature for 30 min in paraformaldehyde solution. The cells were then maintained in 3% H_2O_2 for 5 min; 0.1% Triton-X solution was then dripped onto the cells washed with PBS and maintained for 15 min. After the incubation period, protein blocks were dripped onto the cells and kept in the dark for 5 min. The primary antibody (8-OHdG; dilution ratio, 1/100; cat. no. sc-66036, Santa Cruz Biotechnology, Inc.) was then dropped and incubated at room temperature for 30 min in accordance with the instructions for use. Immunofluorescence secondary antibody was used as a secondary marker (FITC; dilution ratio, 1/500; cat. no. ab6785, Abcam) and kept in the dark for 45 min (room temperature). Subsequently, DAPI with mounting medium (dilution ratio, 1/200; cat. no. D1306, MilliporeSigma) was dripped onto the sections and kept in the dark for 5 min (room temperature), and the sections were closed with a coverslip. The stained sections were examined under a fluorescence microscope (Zeiss Axio; Zeiss AG).

Statistical analysis. The results were calculated as the mean ± standard error. Statistical comparisons between groups were performed using one-way ANOVA with Tukey's HSD test. For statistical analyzes, all calculations were performed using SPSS 20 software (IMP Corp.). A value of $P<0.05$ was considered to indicate a statistically significant difference in all tests. In order to determine the intensity of positive staining from the images obtained as a result of the dyeing; five random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program (Blue edition) (Zeiss AG). Data were statistically defined as the mean and standard deviation (mean ± SD) for % area. The one-way ANOVA test (Fig. 8) was performed to compare positive immune-reactive cells and immune-positive stained areas with the healthy controls. As a result of the test, a value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability. The evaluation was performed in two steps. In the first step, the toxicological effects of boric acid and potassium metaborate on the fibroblast cell line were evaluated. In the second step, the effects of the MIC on the fibroblast cell line were determined. In the present study, after the cells reached the appropriate density (80%), MRSA bacteria were added to the wound line. After 30 min, boric acid and potassium metaborate were added to the culture and the results of cell viability assay after 24 hours were determined (Figs. 2 and 3).

According to the results obtained, cell viability decreased significantly with either boric acid (2,500 to 10,000 μg/ml) and potassium metaborate (1,200 and 2,400 μg/ml) (Fig. 2).

TAC and TOS in the wound model. The results revealed that there was a significant difference between boric acid and potassium metaborate in both the TAC and TOS assays compared to the bacteria control group. Potassium metaborate was more effective (0.3 trolox equiv/mmol/l) than boric acid concerning its antioxidant capacity; the oxidant status was similar (Fig. 4).

LDH and Gr concentrations in the wound model. LDH can be used as a marker of cell death, and Gr marker is a critical molecule in resisting in oxidative stress (Fig. 5). The MRSA

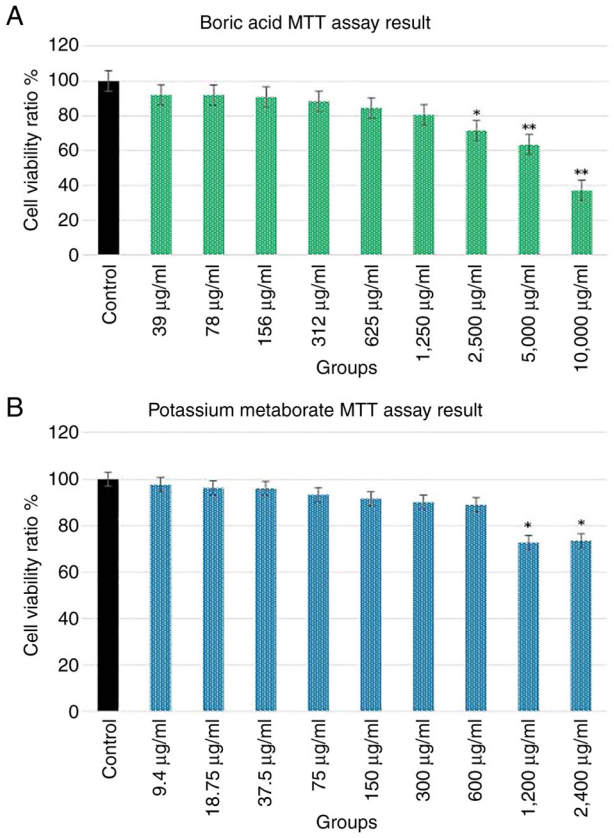

Figure 2. MTT assay results for the fibroblast cell line, methicillin-resistant *Staphylococcus aureus* bacteria co-culture and 24-h (A) boric acid and (B) potassium metaborate treatment. *P<0.05 and **P<0.001, compared to the control group.

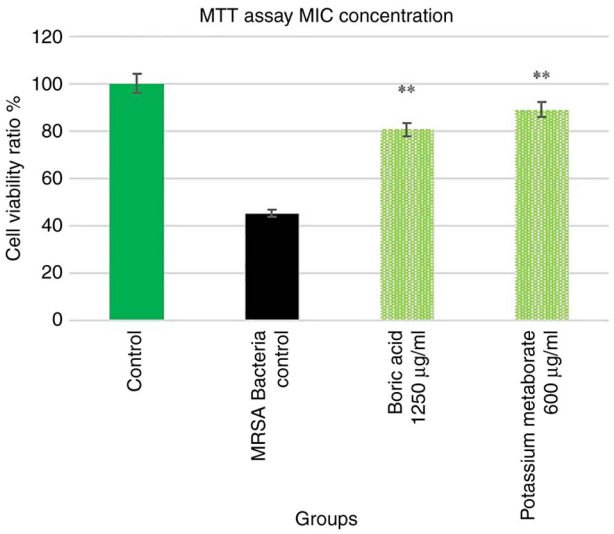

Figure 3. MTT assay results for the fibroblast cell line. The groups were as follows: The control group (only medium), the MRSA bacteria control (fibroblast + MRSA bacteria), MRSA bacteria co-culture and 24 h boric acid 1,250 μg/ml and potassium metaborate 600 μg/ml treatment. **P<0.001, compared to the control group. MRSA, methicillin-resistant *Staphylococcus aureus*; MIC, minimal inhibitory concentration.

control group exhibited an LDH level up to 7- and 2-fold higher than that of the control and treatment groups, respectively. In addition, boric acid and potassium metaborate reduced the

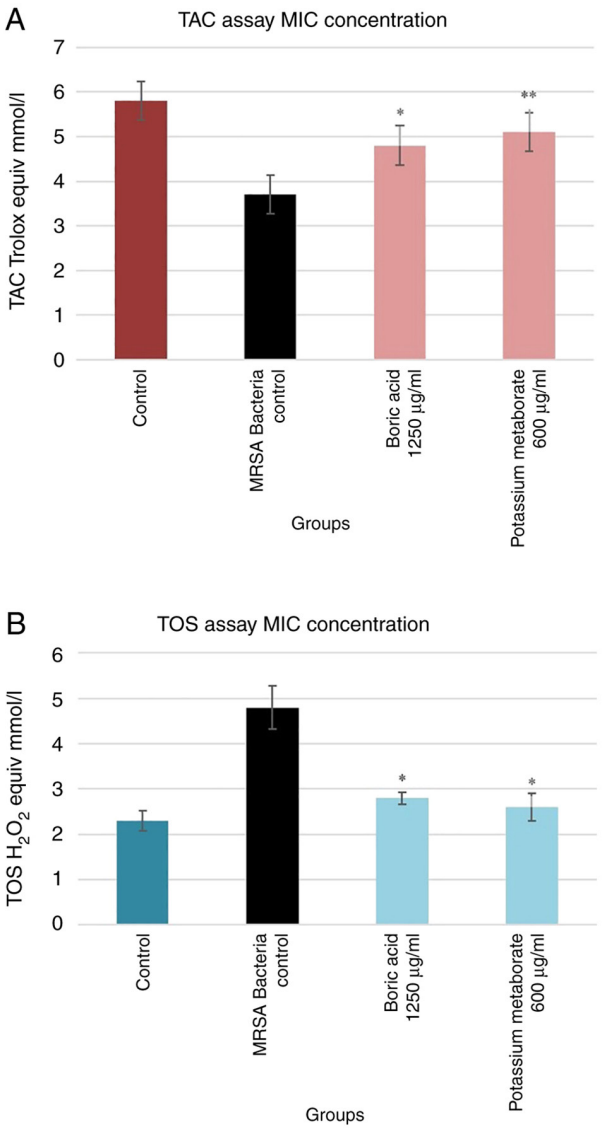

Figure 4. (A) TAC and (B) TOS assays for the fibroblast cell line. The groups were as follows: The control group (only medium), the MRSA bacteria control (fibroblast + MRSA bacteria), MRSA bacteria co-culture and 24 h boric acid 1,250 μg/ml and potassium metaborate 600 μg/ml treatment. *P<0.05 and **P<0.001, compared to the control group. TOS, total oxidant status; TAC, total antioxidant capacity; MRSA, methicillin-resistant *Staphylococcus aureus*.

LDH level to a value similar to that obtained for the control group (the percentage of LDH activity was measured by the ratio [absorbance of the sample/absorbance of maximum activity) x100 (25)]. The results of Gr assay revealed that MRSA decreased the Gr level compared to the control group (26). Potassium metaborate increased the Gr status more effectively (2-fold greater) than boric acid. Although the Gr level increased with boric acid, this increase was not as prominent as that observed in the control group (Fig. 5).

Antibiotic susceptibility. The antibiotic susceptibility results obtained from the automated system VITEK 2 device were interpreted according to the EUCAST criteria (5). The susceptibility to all antibiotics is presented in Table I.

The antibiotic susceptibility of the MRSA bacteria was determined according to the EUCAST criteria. According

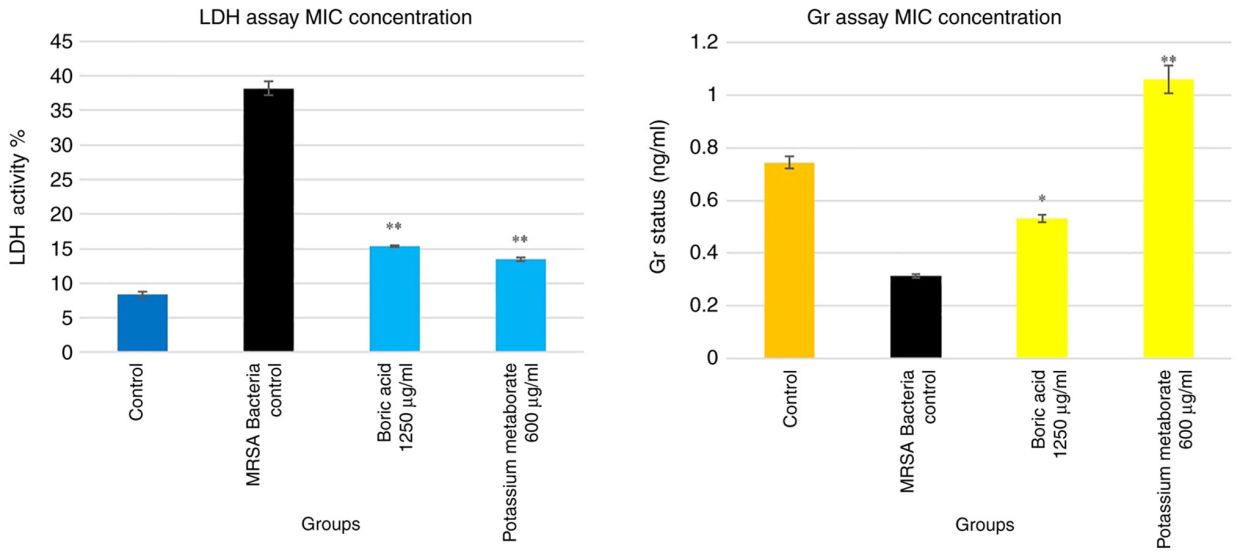

Figure 5. LDH and Gr results for the fibroblast cell line. The groups were as follows: The control group (only medium), the MRSA bacteria control (fibroblast + MRSA bacteria), MRSA bacteria co-culture and 24 h boric acid 1,250 µg/ml and potassium metaborate 600 µg/ml treatment. *P<0.05 and **P<0.001, compared to the control group. LDH, lactate dehydrogenase; Gr, glutathione reductase; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table I. Antibiotic susceptibility.

Antibiotic	S	I	R
Amikacin (30 µg/ml)	X		
Erythromycin (30 µg/ml)			X
Fusidic acid (10 µg/ml)	X		
Gentamicin (10 µg/ml)	X		
Clindamycin (2 µg/ml)			X
Levofloxacin (5 µg/ml)	X		
Linezolid (10 µg/ml)	X		
Penicillin (1 µg/ml)	X		
Ciprofloxacin (5 µg/ml)		X	
Tetracycline (30 µg/ml)			X
Trimethoprim sulfamethoxazole (1.25+23.75 µg/ml)	X		

S, sensitive; I, intermediate; R, resistance.

Table II. Boric acid and potassium metaborate susceptibility.

Boron compounds	S	I	R
Boric acid (20 µg/ml)	X		
Potassium metaborate (20 µg/ml)			X

S, sensitive; I, intermediate; R, resistance.

to these criteria, the bacteria were sensitive to amikacin (30 µg/ml), fusidic acid (10 µg/ml), gentamicin (10 µg/ml), levoflaxacin (5 µg/ml), linezolid (10 µg/ml), penicillin (1 µg/ml), trimethoprim sulfamethoxazole (1.25 +23.75 µg/ml) and ciprofloxacin (5 µg/ml) (intermediate), and were resistant to erythromycin (30 µg/ml), clindamycin (2 µg/ml) and tetracycline (30 µg/ml).

Kirby-Bauer disk diffusion method results. Sterile Paper discs (6 mm in diameter, Oxoid Antibacterial Susceptibility Blank Test Disc) were impregnated with 20 µg/ml and placed in Mueller-Hinton medium. Zone diameters were measured following 24 h of incubation. After 24 h of incubation according to the Kirby-Bauer disk diffusion results, boric acid formed a 25 mm diameter zone, while potassium metaborate formed a 12 mm diameter zone in the antibiogram for MRSA. The generated zone diameter sensitivities are presented in Table II and Fig. 6. The MRSA bacteria were found to be sensitive to boric acid (20 µg/ml), and to exhibit resistance to potassium metaborate (20 µg/ml).

MIC values of boron compounds. The final concentration of the boron compounds was determined as 20 mg/ml. The MIC values were measured by dilution (20-0.039 mg/ml) from the solution. As a result of the dilution, it was determined that potassium metaborate (0.62 mg/ml) and boric acid (2.5 mg/ml) inhibited the growth of bacteria. Boron compounds exhibited an effect on bacteria in these value ranges.

Biofilm assay. Three tests are used to detect the presence of biofilm as follows: In the tube test, the presence of biofilm was detected by residues of the safranin solution. MRSA cones treated with 1% safranin solution were evaluated as strong positive (+++) according to the formation and density of a colored film layer on the inner surface of the tube. The biofilm feature in the tube test is illustrated in Fig. 6.

To quantitatively determine biofilm formation, the optical densities of microplates with ELISA wells were measured at OD 450 - OD 630 OD nm using an ELISA reader. During the test, sterile TSB was examined and evaluated as a negative control. As regards the use of boric acid on the MRSA colonies, the absorbance values were

Table III. The results of the biofilm feature of MRSA with the three methods.

Microorganism type/method	Tube test method	Microplate method	Congo red agar method
MRSA	(+++)	0.315 nm	Dry crystallized black colony
Escherichia coli ATCC 25922	(-)	0.045 nm	White/Pink Colony

MRSA, methicillin-resistant *Staphylococcus aureus*.

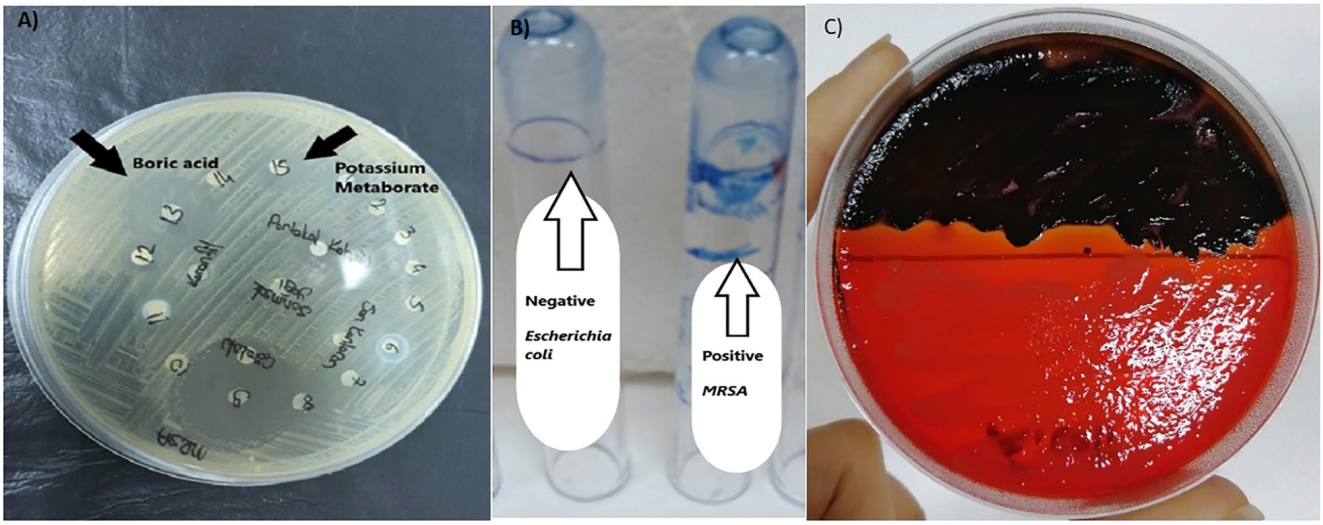

Figure 6. (A) Boric acid and potassium metaborate zone diameters (B) Indication of MRSA tube assay biofilm presence. (C) Congo agar red method, *Escherichia coli*, white/pink colony, MRSA, dry crystallized black colony. MRSA, methicillin-resistant *Staphylococcus aureus*.

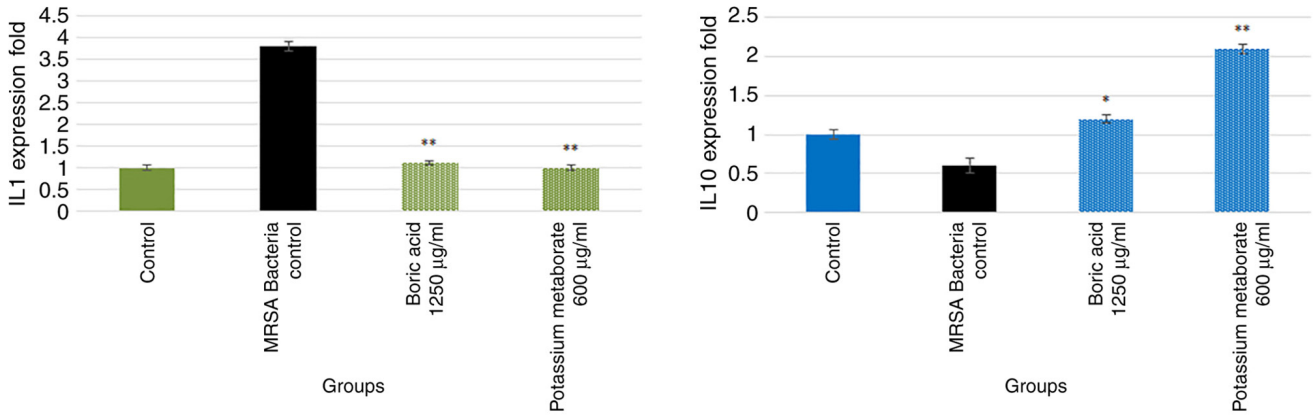

Figure 7. IL-1β and IL-10 gene expression level. *P<0.05 and **P<0.001, compared to the control group. MRSA, methicillin-resistant *Staphylococcus aureus*.

established at a wavelength of 0.315 nm and at a wavelength of 0.131 nm for potassium metaborate. As a negative control, the OD value was determined as 0.045 nm in *E. coli* (Tables III and IV).

Strains that formed dry crystalline black colonies on the surface of the medium as a result of incubation were evaluated as biofilm-positive. The results of the three methods applied to the MRSA colonies are presented in Table III. In addition, morphological images in Congo red medium are illustrated in Fig. 6. The E. coli colony was cultivated as a negative control (Tables III and IV).

IL-1β and IL-10 gene expression. IL-1β levels were determined as a marker of inflammation and IL-10 levels were determined as it has anti-inflammatory properties (Fig. 7). The results revealed an almost 4.5-fold increase in the levels of IL-1β in the bacteria group compared to the control group. However, treatments with boric acid and potassium metaborate significantly reduced IL-1β expression (P<0.001). In addition, the IL-110 level decreased in the bacteria group by almost 0.2-fold; treatment with boric acid increased IL-10 expression level to levels similar to those of the control group. However, potassium metaborate increased the IL-10

Table IV. OD values of MRSA boric acid and potassium metaborate.

Microorganism type/compound	OD value	Positive control	Negative control
MRSA/Boric acid	0.315 nm	0.255 nm	0.216 nm
MRSA/Potassium metaborate	0.131 nm	0.106 nm	0.11 nm
Escherichia coli ATCC 25922/ Boric acid	0.045 nm	0.101 nm	0.048 nm
Escherichia coli ATCC 25922/ Potassium metaborate	0.060 nm	0.075 nm	0.063 nm

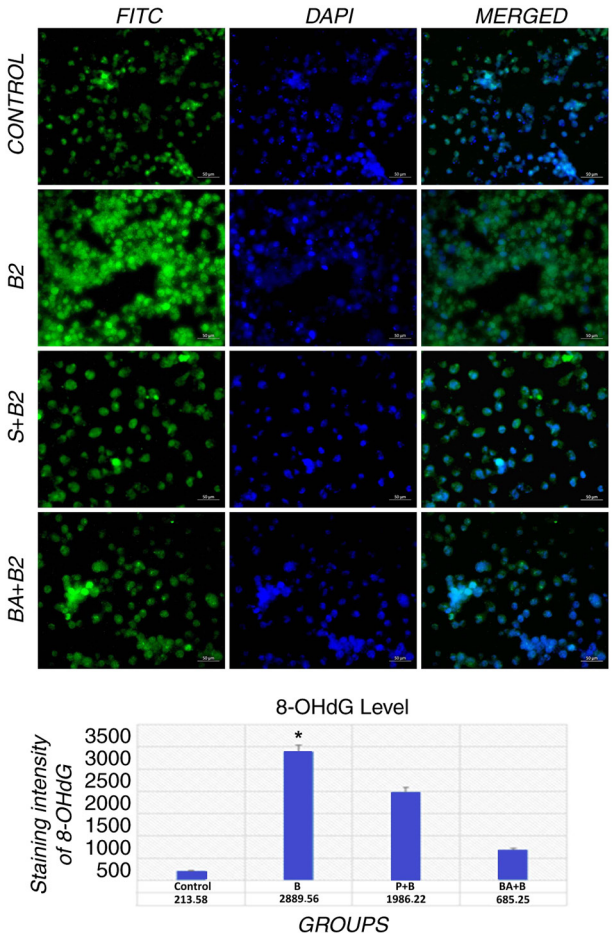

Figure 8. 8-OHdG evaluation. Control, control group; B, MRSA bacteria control; P + B, potassium metaborate + MRSA bacteria; BA + B, boric acid + MRSA bacteria. MRSA, methicillin-resistant *Staphylococcus aureus*. *P<0.05, compared to the control group.

expression level by almost 1-fold compared to the bacteria control group (Fig. 7).

Immunohistochemical evaluation. In mitochondrial and nuclear DNA, 8-hydroxy-2'-deoxyguanosine is the predominant form of free radical-induced oxidative lesions, and thus, it serves as a marker of oxidative stress (23). In line with the previous findings (MTT and LDH test), a significant concentration-dependent increase in the 8-OHdG fluorescent signal was observed in the bacteria control group (Fig. 8).

These results further support the data related to the oxidant abilities of MRSA bacteria already observed in TAS and TOS assays, demonstrating how boric acid and potassium metaborate are able to reduce 8-OHdG, as one of the major products of DNA oxidation and, consequently, of DNA damage accumulation and fibroblast cell death. In particular, a mild-moderate fluorescence signal intensity was observed with boric acid compared to potassium metaborate, while marked signal intensities were observed in the bacteria control group (Fig. 8) (29,30).

Discussion

MRSA strains have begun to exhibit multi-drug resistance with their biofilm structure and virulence factors. This situation causes problems in treatment protocols with conventional antibiotics, forcing the elimination of MRSA infections (24). The World Health Organization (WHO) categorized MRSA strains as a high priority pathogen in 2017 (31). MRSA is the most frequently isolated agent in wound samples, particularly from diabetic foot wound samples. The higher antibiotic concentration (almost 100-fold) of vancomycin and gentamicin used has been shown to prevent the penetration of antibiotics by acting on the biofilm (32,33). As a result of this situation, it is important to investigate molecules to prevent biofilm formation. Thus, in the present study, the antibacterial effects of boron compounds (potassium metaborate and boric acid) were evaluated (34).

In the present study, 600 μg/ml potassium metaborate and 1,250 μg/ml boric acid exhibited a minimal inhibition of MRSA as a result of dilution with the final concentration of 20 mg/ml boron compounds. Yılmaz (27) found the MIC value of 3.80 mg/ml when examining the effect of boric acid and borax on the *S. aureus* ATCC 25923 strain. Similarly, Sayın *et al* (35) found that the MIC value of 3.09 mg/ml when examining the antibacterial and biofilm effects of boric acid on the *S. aureus* ATCC 25923 strain. When common boric acid was compared in the present study, the closest MIC value was found. In the present study, dry black colonies were observed in Congo red medium. Tge OD value was determined at 0.315 and 630 nm using an ELISA reader in tube test (+++) and microplate method.

The inhibition concentration of boron derivatives was applied to the bacteria contaminated wound model (fibroblast wound model with MRSA contamination). In the experiment, the cell viability ratio was determined using MTT assay.

The control group with MRSA contamination exhibited a decreased fibroblast viability ratio by almost 60%. Treatment with boric acid and potassium metaborate inhibited MRSA toxicity and increased cell viability by up to 35 and 45% respectively. By contrast, the LDH level in the boron treatment groups exhibited a decrease. There is an association between LDH activity and TOS. MRSA dominantly led to increased cytotoxicity by inducing cell stress and oxidative damage (was be detected by an increase in LDH and TOS levels, whereas Gr and TAC levels exhibited a notable decrease) as well as biofilm formation. An important advantage of these compounds is their high solubility in water and a relatively good cell permeability. Nisha *et al* (36) demonstrated that a boric acid mouth rinse solution significantly reduced bacteria compared to the control group. The inhibition of MRSA activity in the wound model led to an increased glutathione reductase level. Martínez *et al* (37) demonstrated that MRSA induced DNA fragmentation and apoptosis by increasing oxidative stress. In addition, they demonstrated a decrease in oxidative stress levels, leading to an increase in the cell viability ratio. The present study demonstrated that boron components improved the Gr and TAC levels and also revealed that cells can tolerate stress induced by MRSA bacteria (as a result the cytotoxicity decreases significantly).

In addition, Idiz *et al* (38) demonstrated that boric acid significantly reduced IL-1β levels in rats using hydroalcoholic extract during the liposaccharide-associated acute inflammatory response. Boric acid also increased the IL-10 expression level at the same time. The mechanisms of action of boron components have not yet been fully elucidate; however, their suppressive effects on free radicals and the elevation of the antioxidant capacity have been well-described. Another study also demonstrated that potassium metaborate and boric acid decreased pro-inflammatory cytokine levels (IL-1β) and increased IL-10 levels significantly (39). Another parameter for evaluation oxidative stress is DNA damage.

The present study examined a wound model using the DNA fragmentation marker, 8-OHdG (immunohistochemical staining). The fibroblast intracytoplasmic 8-OHdG expression level was high in the MRSA group ($P<0.05$). The treatment groups exhibited decreased 8-OHdG expression levels, particularly the boric acid group. The DNA fragmentation level decreased significantly compared to the MRSA group. Geyikoglu *et al* (40) demonstrated that boric acid decreased the 8-OHdG expression level in ischemia reperfusion injury by acting as an antioxidant, anti-inflammatory and antiapoptotic agent. The results of TAC, TOS and LDH assays supported the results from the 8-OHdG staining assay (41,42).

A positive finding of the present study is that it demonstrated that boron compounds exhibit antimicrobial activity against MRSA. The present study determined biofilm formation using three methods. However, some limitations of the present study were that although biofilm formation was demonstrated phenotypically, molecular biofilm-forming gene regions were not detected due to unavailable economic conditions.

In conclusion, the results of the present study suggest that boric acid and potassium borate substances can be effective on the tested microorganisms. Further studies on the effects of these microorganisms using various concentrations and treatment durations are required. However, considering that potassium metaborate has not been previously studied, at least to the best of our knowledge, the findings of the present study may shed light on to resistance to these microorganisms and may aid future research into microbial resistance.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DC, ATa, SB, SG, AY, YY, FYe, SY, IB, SK, FYi, AH, OC, DM, GMN, DAS and ATs were involved in the study methodology and analysis, as well as in the writing, reviewing and editing of the manuscript. All authors have read and approved the final manuscript. DC and ATa confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Methicillin-resistant *Staphylococcus aureus* bacteria isolated from the patient sample, for which ethical approval was obtained with the Ataturk University Faculty of Medicine Clinical Research Ethics Committee (decision date, 04.11.2021; decision no. 23; and meeting no. 07) was used in the study. All necessary consents and permissions were obtained for the patient samples used in the study. The ethics committee document obtained from the authors' institution includes all consent forms.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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