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The Antibacterial Effect of Boron Compounds and Evaluation of the Effects on Biofilm Formation in the Infection Model of *Klebsiella pneumoniae* on the HepG2 Cell Line

Klebsiella pneumoniae Enfeksiyon Modelinde HepG2 Hücre Hattında Bor Bileşiklerinin Antibakteriyel Etkisi ve Biyofilm Oluşumuna Etkilerinin Değerlendirilmesi

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Abstract

Aim: *Klebsiella pneumoniae* causes pneumonia, urinary tract infection and bacteremia in immunocompromised patients. *Klebsiella pneumoniae*, which has become more common recently, causes antibiotic resistance as well as pyogenic liver abscesses and hematogenous metastatic spread in humans. Developing antibiotic resistance complicates the treatment of liver infections. In our study, we aimed to evaluate the effect of boron compounds in an infection model created by *Klebsiella pneumoniae* 700603.

Material and Method: The minimum inhibitory concentration, fractional inhibitory concentration and biofilm optical density levels of boron compounds against *Klebsiella pneumoniae* were determined. In these determined dose ranges, a non-toxic dose range for the HepG2 cell line was selected and evaluated by showing with immunofluorescence staining.

Results: We determined the low and high minimum inhibitory concentration values of boron components, sodium perborate monohydrate and etidote, respectively. In addition, sodium perborate monohydrate is also effective on biofilm formation. Our findings have shown that boron compounds are more effective when used in a combination. In the toxicity model created in the cellular study, the boron compounds cytotoxic effect decreased due to their antibacterial effects.

Conclusion: İt seems that boron compounds are effective, and the positive effect increases when used together.

Keywords: HEPG2, *Klebsiella pneumoniae*, minimum inhibitory concentration, boron components

Öz

Amaç: *Klebsiella pneumoniae* bağışıklığı baskılanmış hastalarda pnömoni, idrar yolu enfeksiyonu ve bakteriyemiye neden olur. Son zamanlarda daha sık görülen *Klebsiella pneumoniae*, insanlarda piyojenik karaciğer apselerine ve hematojen metastatik yayılımın yanı sıra antibiyotik direncine de neden olmaktadır. Gelişen antibiyotik direnci karaciğer enfeksiyonlarının tedavisini zorlaştırmaktadır. Çalışmamızda HepG2 hücre hattında *Klebsiella pneumoniae* 700603 tarafından oluşturulan enfeksiyon modelinde bor bileşiklerinin etkisini değerlendirmeyi amaçladık.

Gereç ve Yöntem: *Klebsiella pneumoniae*'ye karşı bor bileşiklerinin minimum inhibitör konsantrasyonu, fraksiyonel inhibitör konsantrasyonu ve biyofilm optik dansite düzeyleri belirlendi. Belirlenen bu doz aralıkların da HepG2 hücre hattı için toksik olmayan doz aralığı seçildi ve immünofloresan boyama ile gösterilerek değerlendirildi.

Bulgular: Bor bileşenleri sodyum perborat monohidrat ve etidot için sırasıyla düşük ve yüksek minimum inhibitör konsantrasyonu değerleri tespit edildi. Ayrıca biyofilm oluşumu üzerine sodyum perborat monohidratın etkili olduğu belirlendi. Bulgularımız, bor bileşiklerinin kombine halinde kullanıldığında daha etkili olduğunu göstermiştir. HepG2 hücre hatında oluşturulan toksisite modelinde, bor bileşiklerinin sitotoksik etkisi antibakteriyel etkilerinden dolayı azalmıştır.

Sonuç: HepG2 hücre hattında oluşturulan *Klebsiella pneumoniae* enfeksiyona karşı bor bileşiklerinin etkili olduğu ve birlikte kullanıldıklarında sinerjistik etkisinin arttığı görüldü.

Anahtar Kelimeler: Bor bileşenleri, HepG2, *Klebsiella pneumoniae*, minimum inhibitör konsantrasyon

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INTRODUCTION

Klebsiella pneumoniae is a well-known opportunistic nosocomial pathogen. Most community-acquired *Klebsiella pneumoniae* infections cause pneumonia and urinary tract infections. However, over the past two decades, a distinctly invasive syndrome causing liver abscesses has been increasingly reported and is emerging as a global disease.[1] Studies have shown that *Klebsiella pneumoniae* strains infect the liver due to the gastrointestinal system. They stated that *Klebsiella pneumoniae* strains isolated from patients with liver abscesses and healthy *Klebsiella pneumoniae* carriers were identical with the same virulence-related genes and median lethal dose values.[2,3] These findings suggest that healthy adults carry virulent strains in their gut. When bacteria migrate through the intestinal epithelium, they can cross the intestinal barrier and form a liver abscess. Fecal-oral transmission, gastrointestinal colonization, and environmental exposure are possible routes of acquisition. A liver abscess develops after *Klebsiella pneumoniae* infiltrates the liver from a patient's intestine via portal circulation.[4] In addition, virulence factors in *Klebsiella pneumoniae*, capsular serotype, mucoviscosity-associated gene A (magA), rmpA and aerobactin are important in the formation of liver abscesses.[4,5]

Klebsiella pneumoniae strains expressing the capsular type K1 or K2 antigen are virulent. These serotypes have a high prevalence of resistance to phagocytosis and intracellular killing by neutrophils and bactericidal complements in a patient's serum. Although *Klebsiella pneumoniae* serotypes K1 and K2 isolated from patients with liver abscesses usually show hypermucoviscosity, they are known to be an indicator of capsular polysaccharide expression, which is related to resistance to phagocytosis.[5] magA has been identified as the gene that causes *Klebsiella pneumoniae* liver abscess and septic metastatic complications.[6] RmpA aids in capsule synthesis in addition to magA and capsular serotype K1/K2.[7] All strains of *Klebsiella pneumoniae* that cause liver abscesses and abscesses at other sites regulate rmpA capsular polysaccharide synthesis.[8] Aerobactin, a type of siderophore, is an iron chelator that increases the virulence of *Klebsiella pneumoniae* 100-fold and is an important factor for pathogenicity in *Klebsiella pneumoniae*. Aerobactin genes play an important role in the virulence of *Klebsiella pneumoniae* in combination with rmpA.[9]

These virulence factors have an important place in the formation of resistance against antibiotics. The World Health Organization has published a list of antibiotic-resistant bacteria that seriously threaten society. There is an urgent need for new classes of antibiotics with new mechanisms of action against *Klebsiella pneumoniae* on this list.[10-12] Boron compounds are single-active site-directed serine-type β-lactamase inhibitors that are not based on a β-lactam structure. The inhibitory activity of these modules has been extensively studied against various potent β-lactamases. Studies have shown that different boron compounds reversibly and rapidly inhibit C class AmpC enzymes, various A class β-lactamases, such as Bacillus cereus chromosomal penicillinase, and some CTX-M-type enzymes. Since the glycyl-boronic acid compound does not show inhibition of AmpCs alone, compounds with good activity in the β-lactamase classes are scarce. They are important inhibitors for treating infections caused by β-lactamase-producing bacteria.[13-15] In light of this information, we aimed to evaluate the synergistic effect of boron compounds and the presence of biofilms in infections caused by *Klebsiella pneumoniae*.

MATERIAL AND METHOD

Reagents

Etidote (disodium octaborate tetrahydrate), Sodium perborate monohydrate (SPM), Zinc borate (ZB), Mueller-Hinton broth, tryptic soy broth, Dulbecco's modified Eagle's medium (DMEM), phosphate buffer solution (PBS), fetal calf serum (FCS), antibiotic antimitotic solution (100×), L glutamine, trypsin–EDTA, paraformaldehyde and ethanol were obtained from Sigma Aldrich (St. Louis, MO, USA).

Bacterial Strain

Klebsiella pneumoniae 700603 was used in our study. The isolate was identified by conventional methods and an automated system (Phoenix, Becton Dickinson, USA). Suspensions equivalent to strain 0.5 McFarland turbidity were prepared.

Experimental design

The experiment was designed in two different parts.

The first part evaluated the minimal inhibitory concentration (MIC) of Etidote (39, 78, 156, 312, 625, 1250, 5000, and 10000 µg/mL), sodium perborate monohydrate (39, 78, 156, 312, 625, 1250, 5000, and 10000 µg/mL), and zinc borate (9.38, 18.75, 37.50, 75, 150, 300, 600, 1200, and 2400 µg/mL). The antibacterial effects of etidote, sodium perborate monohydrate, and zinc borate were analyzed according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS). *Klebsiella pneumoniae* 700603 strains were used for the test. The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation. MICs were defined as the lowest concentration of etidote, sodium perborate monohydrate, and zinc borate, inhibiting the visible growth of the bacteria. The MICs of etidote, sodium perborate monohydrate, and zinc borate were conventionally determined in triplicate for each strain by the microdilution broth method as described by the NCCLS. Serial dilutions of etidote, sodium perborate monohydrate and zinc borate were prepared in microdilution with concentrations ranging between 1000 µg/mL (40 mM) and 0.97 µg/mL (40 mM). A total of 125 µg/mL etidote, 62.5 µg/mL sodium perborate monohydrate, and 31.25 µg/mL zinc borate showed minimal inhibition of *Klebsiella pneumoniae* 700603.

In the second step, a *Klebsiella pneumoniae* 700603 model was induced using the HepG2 cell line; SPM 62,5 µg/mL + Etidote 125 µg/mL, SPM 62,5 µg/mL + ZB 31,25 µg/mL and ZB 31,25 µg/mL + Etidote 125 µg/mL were applied for 24 h treatments. It was preferred because it did not show toxicity in these dose ranges. After the end of the experiment, MTT, TAC, TOS, Gr, LDH and immunohistochemistry analyses were performed.

Cell cultures

HepG2 cell (HB-8065 ATCC) cultures were obtained from the Department of Medical Pharmacology of Ataturk University (Erzurum, Turkey). Briefly, the cells were resuspended in fresh medium (Dulbecco's modified Eagle's medium, DMEM), 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin, streptomycin, and amphotericin B). Then, the cells were seeded in 24-well plates (Corning, USA) and stored in an incubator (5% CO_2; 37°C).[16] After gaining an 85% confluence ratio, the model was performed using a 100 µL yellow pipet tip; the McFarland 0.5 scale bacterial suspension was added to the cell culture. After 30 min of treatment with the HepG2 cell line, SPM 62,5 µg/mL + Etidote 125 µg/mL, SPM 62,5 µg/mL + ZB 31,25 µg/mL and ZB 31,25 µg/mL + Etidote 125 µg/mL were applied for 24 h.

MTT Assay

At the end of the two-part experiment (after 24 h of treatment with boric acid and potassium metaborate), 10 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well plate, and the samples were incubated for 4 h; 100 µL of DMSO solution was incorporated into all wells to dissolve formazan crystals. The optical density of the solutions was read at 570 nm using a Multiskan™ GO Microplate Spectrophotometer reader.[17,18]

Immunofluorescence

Cells cultivated in cell culture were incubated for 30 min in paraformaldehyde solution. The cells were then incubated in 3% H_2O_2 for 5 min. A 0.1% Triton-X solution was dripped onto the cells, washed with PBS and left for 15 min. After incubation, protein blocks were dripped onto the cells and kept in the dark for 5 minutes. Then, the primary antibody (8-OHdG cat no: sc-66036, dilution ratio: 1/100 US) was added dropwise and incubated following the instructions for use. Immunofluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785, dilution ratio: 1/500, UK) and incubated in the dark for 45 minu. Then, the second primary antibody (H2A. X Cat No: I 0856-1, dilution ratio: 1/100, US) was dripped onto the tissues and incubated per the instructions. Immunofluorescence secondary antibody was used as a secondary marker (Texas Red Cat No: ab6787, dilution ratio: 1/1000 UK) and kept in the dark for 45 min. Then, DAPI with mounting medium (Cat no: D1306, dilution ratio: 1/200 UK) was dripped onto the sections and kept in the dark for 5 min, and the sections were closed with a coverslip. The stained sections were examined under a fluorescence microscope (Zeiss AXIO GERMANY).

Bacterial Production

The bacterial stock of *Klebsiella pneumoniae* 700603 was added to 100 µL of tryptic soy broth (TSB) medium, and after 24 h of incubation at 37°C and 150 rpm, its production was carried out. Then, 200 µL of the growth medium was taken and inoculated into fresh TSB, and the stock medium was made ready for the study.

Minimum Inhibitory Concentration

The MIC values of sodium perborate monohydrate (SPM), zinc borate (ZB), and etidote compounds against *Klebsiella pneumoniae* 700603 were determined using the microdilution method. Dose ranges were determined as 1000-0.97 µg/mL. Müeller-Hinton broth (MHB) medium was inoculated into 96-well plates to which 180 µL of each dilution was added. Then, 20 µL of *Klebsiella pneumoniae* 700603 (10^6CFU/mL) was added to each well and incubated at 37°C.

Biofilm Analysis

A total of 180 µl of the compounds whose MIC value was determined, prepared with TSB medium, was inoculated into a flat-bottomed 96-well plate. Glucose-enriched TSB medium was used as a negative control, and the *Klebsiella pneumoniae* 700603 strain was used as a positive control. Then, 20 µL (10^6 CFU/mL) of the *Klebsiella pneumoniae* 700603 strain was inoculated into each well except the negative well. It was incubated at 37°C for 48 h. Biofilm analysis was performed in 3 repetitions.

The effects of SPM, ZB, and Etidote on *Klebsiella pneumoniae* 700603

The most effective MIC concentrations of SPM, ZB, and Etidote compounds were prepared in combination. In the analysis performed, similar to the biofilm evaluation test principle, the *Klebsiella pneumoniae* 700603 strain was inoculated into MHB medium enriched with glucose and incubated at 37°C for 48 h. Bacterial growth was expected. In addition, the medium was made fresh by adding TSB medium to the plates at 24-36 h intervals. After 48 h, the liquid in the plates was evacuated. A 200 µL aliquot of glucose-enriched culture medium containing TTC (5 mg/mL) was added to each well and incubated at 37°C for 3-4 h. The intensity of the red color at the end of the resulting test was considered an indicator of viable cell number and was measured at 490 nm. The results were compared with controls. The test was repeated in triplicate.

Microdilution panels

The solutions were prepared by calculating the final concentrations of SPM, ZB, and Etidote compounds on the prepared panels. Intermediate dilutions with a concentration of four times the final concentration desired in the well were prepared. Then, 100 µL of TSB medium was dispensed into all wells. First, 100 µL of SPM was diluted in half and dispersed, and then 100 µL was added to the wells diluted sequentially with ZB and Etidote 1000 µg/mL. The medium was prepared

as a negative control, and bacterial wells were prepared as a positive control. Except for the negative control well, antimicrobial agents (5 µl) were dispensed into the plates. This process was repeated for the other ZB and Etidote, and 3 repetitions were applied.

Fractional Inhibitor Concentration Index-Combination (FIC)

The FIC for each drug is determined by dividing the MIC of each drug when used in combination by the MIC of each drug used alone. The FIC index quantifies drug interactions and the value is used to determine interactions between tested drugs. It was applied according to the FIC index formula used to determine the effectiveness of the combinations. The results were determined according to the formula.

A: Antimicrobial 1 used in combination
B: Antimicrobial 2 used in combination
Calculation of the FIC index:
FIC A: MIC numerical value of An in the presence of B/MIC numerical value of A alone
FIC B: MIC numerical value of B in the presence of A/MIC numerical value of B alone
Σ FIC index FIC A + FIC B
Σ FIC index ≤ 0.5: Synergism
Σ FIC index >0.5 and <1: additive
Σ FIC index ≥1 and 4 ≤: ineffective (indifference)
Σ FIC index >4: antagonism

RESULTS

Microbiology Analysis

Minimal inhibitory concentrations (MICs) were determined at concentrations of SPM +Etidote 31.25 µg/ml+125 µg/ml, SPM +ZB 31.25 µg/ml+62.5 µg/ml and ZB+Etidote 62.5 µg/ml+125 µg/ml. The dose ranges in which boron compounds are synergistic, ineffective, additive and antagonism against *Klebsiella pneumoniae* are shown in **Figures 1A**, **1B** and **1C**, respectively.

The optical density (570/OD) results of the combinations made with the microdilution plate method are summarized in **Figure 2**. **Figure 2A**. Etidote 1024 µg/ml+SPM 64 µg/ml concentrations were found to have the highest effect on biofilms. **Figure 2B** shows that the highest effect on biofilm formation was detected at Etidote 1024 µg/mL+ZB 512 µg/mL. In **Figure 2C**, the highest effect on biofilm formation was detected at the ZB 512 µg/ml+SPM 64 µg/ml concentration.

MTT Assay

We evaluated the toxicological effects of ZB, SPM, and etidote on the HepG2 cell line. Our results revealed that cell viability did not decrease significantly for SPM + Etidote or SPM + ZB (**Figure 3**).

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. In line with previous findings, a significant dose-dependent increase in the 8-OHdG fluorescent signal was observed in the bacterial control group (**Table 1**).

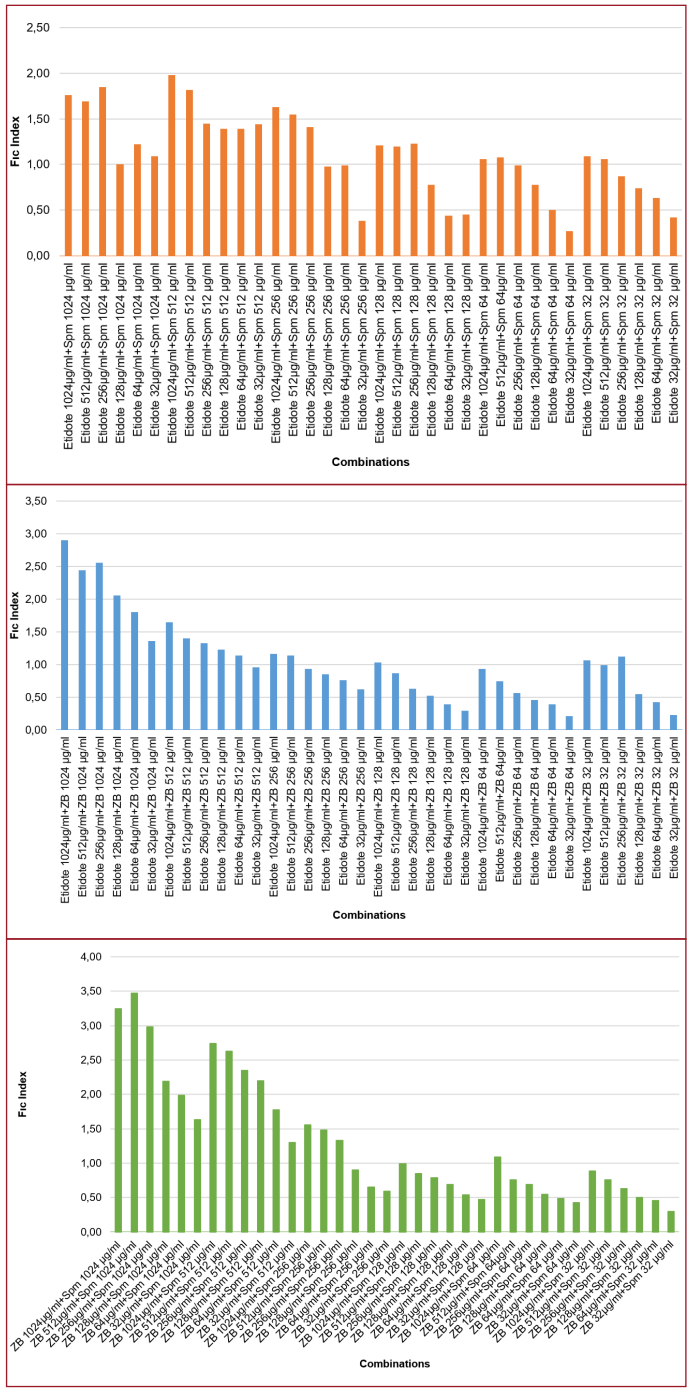

Figure 1. Boron compound FIC index results. A) Etidote+SPM combination FIC index, B) Etidote+ZB combination fix index, C) ZB+SPM combination fix index. Value ranges of boron combinations corresponding to Σ FIC index ≤ 0.5: synergy, >0.5 and <1: additive and ≥1 and 4 ≤: ineffective (indifference).

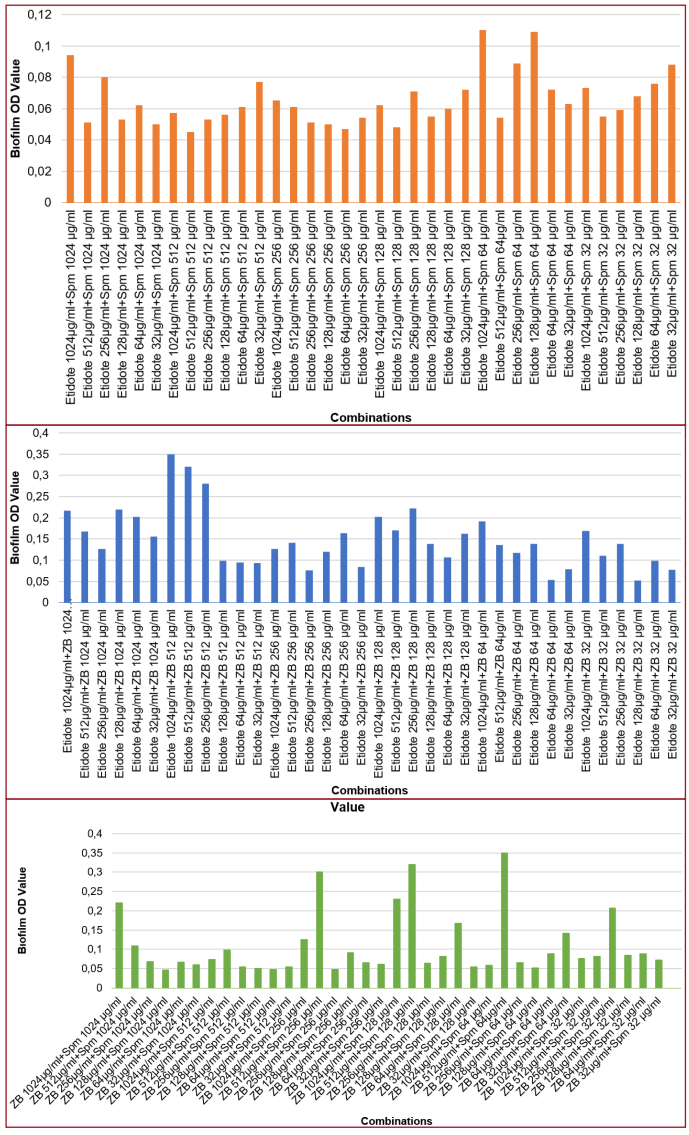

Figure 2. Biofilm OD Results. A) Etidote+SPM, B) Etidote+ZB, C) ZB+SPM biofilm OD values. The minimum and maximum OD values of Etidote+SPM, Etidote+ZB, and ZB+SPM biofilms ranged at 570 OD.

Table 1. Statistical analysis of immunofluorescent staining results.

	8-OHdG	H2A.X
Control	23.38±5.34[a]	19.26±4.58[a]
SPM+Etidote	30.44±5.86[a]	27.39±5.08[a]
SPM+ZB	29.88±6.24[a]	25.15±4.38[a]
ZB+Etidote	44.16±3.89[b]	38.26±4.74[b]

These results further support the data related to the oxidant abilities of *Klebsiella pneumoniae* bacteria already observed in MTT assays demonstrating how SPM 62,5 µg/ml + Etidote 125 µg/ml, SPM 62,5 µg/ml + ZB 31,25 µg/ml, able to reduce 8-OHdG, is one of the major products of DNA oxidation and consequently of DNA damage accumulation and HepG2 cell death. In particular, mild fluorescent signal intensity was observed at SPM 62,5 µg/ml + Etidote 125 µg/ml, SPM 62,5 µg/ml + ZB 31,25 µg/ml and mild-moderate ZB 31,25 µg/ml + Etidote 125 µg/ml compared to the control group (**Figure 4**).

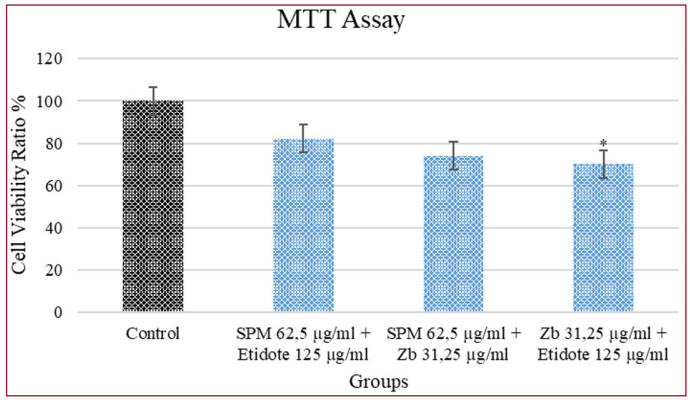

Figure 3. MTT assay results for the HepG2 cell line, Control group (received only medium), *Klebsiella pneumoniae* bacteria coculture for 24 h SPM 62,5 µg/ml + Etidote 125 µg/ml, and SPM 62,5 µg/ml + ZB 31.

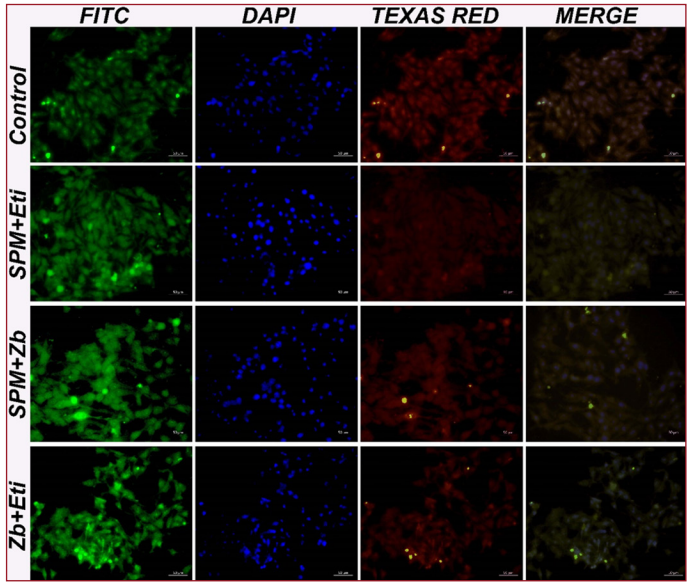

Figure 4. Cell lines, 8-OHdG expression (FITC) and H2A. X expression (Texas Red), IF, Bar: 50 µm.

Statistical Analysis

The results were calculated as the mean ± standard error. Statistical comparisons between groups were calculated using one-way ANOVA and Tukey's LSD method. All calculations were performed using SPSS 20 software for statistical analysis, and $P<0.05$ was considered a significant difference in all tests.

DISCUSSION

Klebsiella pneumoniae is a common opportunistic pathogen that frequently causes nosocomial infections, including pneumonia, meningitis, and bloodstream and urinary tract infections. In addition, *Klebsiella pneumoniae* can potentially cause community-associated infections, such as liver abscesses, endophthalmitis, and meningitis, in healthy individuals.

In this study, we aimed to evaluate the synergistic effect of boron compounds and their effect on biofilms in an infection model induced by *Klebsiella pneumoniae* 700603

on the HepG2 liver cell line. In addition to potential use as β-lactamase inhibitors in studies evaluating the synergistic activity of boron compounds and antibiotics, boron compounds have been used for the phenotypic detection of plasmid-mediated AmpC enzymes. Tests using cefoxitin discs and boronic acid compounds, mainly 3′-aminophenylboronic acid (APBA), successfully detect AmpC enzymes in organisms that do not classically produce these enzymes.[19,20]

These tests using extended-spectrum cephalosporins combined with clavulanic acid have helped detect isolates harboring both ESBLs and AmpCs.[21] A tentative observation of the inhibitory effect of boric acid on *Klebsiella pneumoniae* carbapenemase (KPC) was made by Pasteran et al. During the evaluation of a chromogenic assay that detects s), two KPC-2-producing isolates showed synergy between APBA and cefotaxime, ceftazidime, or carbapenems.[22]

After the first observations of the inhibitory activity of boric acid on KPC, specific phenotypic tests were developed to detect KPCs. This investigation initially revealed that KPC production in isolates that did not produce AmpC-type enzymes was associated with positive combined boronic acid disc tests using cefamycins and cefotaxime as substrates, specifically cefepime and carbapenems. The synergy of phenylboronic acid (PBA) with these antibiotics has been applied to the phenotypic identification of the first KPC-producing isolate in Greece.[23]

Further evaluation with an extensive collection of KPC-producing isolates showed an apparent synergistic effect between PBA and carbapenems, revealing an enhanced interaction of the PBA fragment with the active site serine residue of class A KPC β-lactamase.[24]

Combined disc tests were considered positive when the addition of PBA to a β-lactam disc resulted in an enlargement of ≥5 mm in diameter of the growth inhibition zone compared to the zone of inhibition around the β-lactam disc alone. In these studies, combined disc tests using 400 µg PBA with and without cefepime, imipenem, or meropenem demonstrated the highest sensitivity and specificity for detecting KPC enzymes.[23,24]

Our study examined the synergistic effects of boron compounds since resistance to antimicrobials applied in pathogen-oriented treatments leads to alternative searches. Boron compounds have been reported to inhibit this enzyme, especially by binding to the serine residue, especially with the overexpression of β-lactamase classes. In studies conducted to discover new compounds that can be applied with β-lactam antibiotics, boron compounds have also taken their place in the literature.[25]

In the checkerboard study, we examined the interaction of these inhibitors with each other, and a synergistic effect of 32 µg/ml Etidote + 32 µg/ml SPM was detected. SPM: Due to the use of SPM: sodium perborate monohydrate, especially in wound cleaning, in our study, nontoxic appropriate dose ranges were determined, and its combination with etidote was made. Etidote is especially important for using plants in the soil to yield nitrogen and phosphorus sources. In addition, in a study, etidote used together with gelatin was shown to inhibit the growth of *P. aeruginosa*, *S. aureus* and *C. albicans* pathogens.[26]

CONCLUSİONS

In microbiological analyses of boron compounds, FIC and MIC concentrations were high. Statistical significance was determined in the combination of ZB+Etidote against *Klebsiella pneumoniae* in cell culture compared to other groups. Although the results are promising for us, we think that after a more comprehensive examination of the mechanisms of action of these compounds, we should consider whether they will be antimicrobial candidates after in vivo studies on the pathogen and the host.

ETHICAL DECLARATIONS

Ethics Committee Approval: The standard strain was used in this study. No ethical approval is required.

Informed Consent: Since this study was not conducted on patients, a consent form is not required.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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