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Evaluation of the Effect of the Combination of Boron Compounds on Chronic Liver Disease

Bor Bileşikleri Kombinasyonunun Kronik Karaciğer Hastalığı Üzerindeki Etkisinin Değerlendirilmesi

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Abstract

Aim: *Enterococcus faecalis* has surface adhesion proteins that enable it to attach to human intestinal and vaginal tissue cells with antibiotic-resistant strains in patients. Due to these properties, boron and its derivatives are preferred as therapeutic agents due to their antibacterial, antifungal, antiparasitic and anti-inflammatory activities. In this study, we aimed to evaluate the synergistic effect of boron compounds and their effect on biofilms in an infection model created with *Enterococcus faecalis* 29212 on the HepG2 liver cell line.

Material and Method: Synergistic of boron and boron compounds and biofilms minimum inhibitor concentration, fractional inhibitor HepG2 cell analyzes with concentration and biofilm studies was made and evaluated.

Results: It was determined that sodium perborate monohydrate+zinc borate had the lowest values as a result of the minimum inhibitory concentration and fractional inhibitor concentration studies. It has also been shown that these doses reduce cytotoxic effects. In addition, 32 µg/ml Etidote+256 µg/ml Sodium perborate monohydrate showed the highest biofilm effect.

Conclusion: Biofilm formation of Enterococcus faecalis by boron compounds effectively reduce and cause the death of bacteria we showed.

Keywords: Biofilm, boron compounds, fractional inhibition concentration, cell culture, synergistic effect

Öz

Amaç: *Enterococcus faecalis*, hastalarda antibiyotiğe dirençli suşlarla insan bağırsak ve vajinal doku hücrelerine bağlanmasını sağlayan yüzey yapışma proteinlerine sahiptir. Bu özelliklerinden dolayı bor ve türevleri antibakteriyel, antifungal, antiparaziter ve antifungal aktivitelerinden dolayı terapötik ajanlar olarak tercih edilmektedir. Bu çalışmada, HepG2 karaciğer hücre hattı üzerinde *Enterococcus faecalis* 29212 ile oluşturulan bir enfeksiyon modelinde bor bileşiklerinin sinerjistik etkisini ve biyofilmler üzerindeki etkisini değerlendirmesi amaçlandı.

Gereç ve Yöntem: Bor ve bor bileşiklerinin sinerjistik ve biofilmler üzerine etkisi minimum inhibitör konsantrasyonu, fraksiyonel inhibitör konsantrasyonu ve biyofilm çalışmaları ile HepG2 hücre analizleri yapılarak değerlendirildi.

Bulgular: Minimum inhibitör konsantrasyonu ve fraksiyonel inhibitör konsantrasyonu çalışmaları sonucunda sodyum perborat monohidrat+çinko boratın en düşük değerlere sahip olduğu belirlendi. Ayrıca bu dozların sitotoksik etkileri azalttığı da gösterilmiştir. Ayrıca en yüksek biyofilm etkisini 32 µg/ml Etidot+256 µg/ml Sodyum Perborat Monohidrat gözlendi.

Sonuç: Bor bileşiklerinin *Enterococcus faecalis*'in biyofilm oluşumunu etkili bir şekilde azalttığı ve bakterilerin ölümüne neden olduğunu gösterdik.

Anahtar Kelimeler: Biyofilm, bor bileşikleri, fraksiyonel inhibisyon konsantrasyonu, hücre kültürü, sinerjik etki

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INTRODUCTION

Enterococcus faecalis is known to be abundant in the microbiota of patients with chronic liver disease associated with hepatitis C virus. *Enterococcus faecalis* is a gram-positive pathogen that forms biofilms and shows resistance to many antibiotics. It causes infections that are difficult to treat after liver transplantation in patients with chronic liver failure.[1] In addition, antibiotic-resistant *Enterococcus faecalis* has acquired intrinsic resistance to antimicrobial agents such as beta-lactams and aminoglycosides and resistance to glycopeptides, quinolones, tetracyclines, macrolides and streptogramin through the horizontal transfer of elements such as transposons and plasmids or resistance genes.[2] The pathogenic properties of *Enterococcus faecalis* often include biofilm formation with an increasing burden of antimicrobial resistance among the most highly pathogenic nosocomial infections. provides structural integrity.[3] Bacterial biofilms support the survival and persistence of infecting microbes as they facilitate fate defense against the host immune response.[4] *Enterococcus faecalis* encodes several factors that contribute to biofilm formation, including 2 sortase enzymes, SrtC and SrtA, which polymerize and attach the pili associated with endocarditis and biofilm formation.[5-7] This pili assists the adhesion of *Enterococcus faecalis* to surfaces, which is essential during the early stages of biofilm formation in vitro and in vivo during cathepsin-associated urinary tract infection.[8] Other biofilm-associated factors that bind to the cell wall by SrtA include Ace, aggregation agent, and Esp. *Enterococcus faecalis* must also defeat host defenses to establish infection. *Enterococcus faecalis* can modulate and evade the host immune response in a number of settings. Biofilm formation, along with the expression of the SrtA substrate aggregation agent, can promote the survival of *Enterococcus faecalis* within macrophages and neutrophils. The multipeptide resistance factor (MprF) protein of *Enterococcus faecalis* confers resistance to antimicrobial peptides via electrostatic repulsion and is important for both neutrophil-mediated clearance and survival in epithelial cells and macrophages in a variety of gram-positive bacteria.[9-11] The inadequacy of antibiotics used in the treatment against bacterial resistance and biofilms has led to a need for better alternatives. The unique electronic properties of boron that allow it to act as a transition state mimetic for tetrahedral intermediate peptide bond cleavage observed in proteolytic enzymes have attracted increased attention over the past few years as potential drugs. In addition to boronic acids discussed by numerous researchers, benzoxaborols, a class of compounds in which the boron atom is incorporated into a heteroaromatic ring system, have provided a number of interesting anti-inflammatory, antifungal, antiparasitic and antibacterial drug candidates.[12-14] In light of this information, we aimed to evaluate the synergistic effect of boron compounds and their effect on biofilms in the infection model created with *Enterococcus faecalis* 29212 on the HepG2 liver cell line.

MATERIAL AND METHOD

Reagents

Etidote (disodium octaborate tetrahydrate), sodium perborate monohydrate, zinc borate, 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

Enterococcus faecalis 29212 was used in our study. The isolate was identified by conventional methods and an automated system (Phoenix, Becton Dickinson, USA). Suspension equivalent to a strain of 0.5 McFarland turbidity were prepared.

Bacterial Production

The bacterial stock of *Enterococcus faecalis* 29212 was added to 100 µl of tryptic soy broth (TSB) medium, and its production was carried out after 24 hours of incubation at 37°C and 150 rpm. 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 Inhibition Concentration Values

The MIC values of sodium perborate metahydrate (SPM), zinc borate (ZB), and Etidote compounds against *Enterococcus faecalis* 29212 were determined using the microdilution method. The dose range was determined to be 1024-0.97 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 *Enterococcus faecalis* 29212 (10^6 CFU/ml) was added to each well and incubated at 37°C. After 24 hours, TTC water-soluble salt solution, a biological indicator, was added to each well (5 mg/ml), and the plates were incubated for 2-3 hours.[3]

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 *Enterococcus faecalis* 29212 strain was used as a positive control. Then, 20 µl (10^6 CFU/ml) of the *Enterococcus faecalis* 29212 strain was inoculated into each well except the negative well. The cells were incubated at 37°C for 48 hours. Biofilm analysis was performed in 3 repetitions.[3]

Combination Application of *Enterococcus faecalis* 29212 with SPM, ZB, Etidote Compounds

The most effective MIC concentrations of SPM, ZB, and Etidote compounds were prepared in combination with each other. In the analysis performed similar to the biofilm evaluation test principle, the *Enterococcus faecalis* 29212 strain was inoculated into MHB medium enriched with glucose and incubated at 37°C for 48 hours. Bacterial growth

was expected. In addition, the medium was made fresh by adding TSB medium to the plates at 24-36 hour intervals. After 48 hours, the liquid in the plates was evacuated. Then, 200 µl of glucose-enriched culture medium containing TTC (5 mg/ml) was added to each well and incubated at 37°C for 3-4 hours. 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 applied as 3 repetitions.[3]

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, which were diluted sequentially with ZB, Etidote 1000 µg/ml. 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 applied as 3 repetitions.[3]

Fractional Inhibitor Concentration Index-Combination (FIC)

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: synergy
Σ FIC index >0.5 and <1: additive
Σ FIC index ≥1 and 4 ≤: ineffective (indifference)
Σ FIC index >4: antagonism was accepted as.

Cell cultures

For our study, HepG2 cell (HB-8065 ATCC) cultures were obtained from the Department of Medical Pharmacology of Atatürk 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).[15] After gaining an 85% confluence ratio, the model was established by using a 100 µl yellow pipet tip; according to the McFarland 0.5 scale, a bacterial suspension was then 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.[16]

Immunofluorescence

Cells cultivated in cell culture were incubated for 30 minutes in paraformaldehyde solution for 30 minutes. The cells were then incubated in 3% H_2O_2 for 5 minutes. A 0.1% Triton-X solution was dripped onto the cells washed with PBS and left for 15 minutes. After the incubation period, 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 dropped and incubated in accordance with the instructions for use. Immunofluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/500, UK) and incubated in the dark for 45 minutes. Then, the second primary antibody (H2A. X Cat No: I 0856-1, Dilution Ratio: 1/100, US) was dripped onto the tissues and incubated in accordance with the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (Texas Red Cat No: ab6787 Diluent Ratio: 1/1000 UK) and incubated in the dark for 45 minutes. Then, DAPI with mounting medium (Cat no: D1306 Dilution Rate: 1/200 UK) was dripped onto the sections and kept in the dark for 5 minutes, and the sections were closed with a coverslip. The stained sections were examined under a fluorescence microscope (Zeiss AXIO GERMANY).

RESULTS

Microbiology Analysis

Minimal inhibition concentrations (MICs) were determined at concentrations of SPM 31.25 µg/ml+Etidote 125 µg/ml, SPM 31.25 µg/ml+ZB 62.5 µg/ml and ZB 62.5 µg/ml+Etidote 125 µg/ml. **Figure 1A**, Etidote 32 µg/ml+SPM 512 µg/ml, Etidote 32 µg/ml+SPM 128 µg/ml, Etidote 64 µg/ml+SPM 64 µg/ml, Etidote 32 µg/ml+SPM 64 µg/ml in A, Synergistic effect ≤ 0.5: detected at concentrations of Etidote 64 µg/ml+SPM 32 µg/ml and Etidote 32 µg/ml+SPM 32 µg/ml. If additive effect (>0.5 and <1); Etidote 128 µg/ml+SPM 512 µg/ml, Etidote 64 µg/ml+SPM 512 µg/ml, Etidote 64 µg/ml+SPM 256 µg/ml, Etidote 32 µg/ml+SPM 256 µg/ml, Etidote 64 µg/ml+SPM 128 µg/ml, Etidote 128 µg/ml+SPM 64 µg/ml, Etidote 256 µg/ml+SPM 32 µg/ml, Etidote 128 µg/ml+SPM 32 µg/ml were detected in doses, while the others were found to be ineffective.

In **Figure 1B**, Etidote 32 µg/ml+ZB 512 µg/ml, Etidote 64 µg/ml+ZB 256 µg/ml, Etidote 128 µg/ml+ZB 128 µg/ml, Etidote 64 µg/ml+ZB 128 µg/ml, Etidote 32 µg/ml+ZB 128 µg/ml, Etidote 64 µg/ml+ZB 64 µg/ml, Etidote 32 µg/ml+ZB 64 µg/ml and Etidote 32 µg/ml+ZB 32 µg/ml concentrations Synergistic effect ≤ 0.5: detected. Etidote 64 µg/ml+ZB 1024 µg/ml, Etidote 32 µg/ml+ZB 1024 µg/ml, Etidote 128 µg/ml+ZB 512 µg/ml, Etidote 64 µg/ml+ZB 512 µg/ml, Etidote 256 µg/ml+ZB 256 µg/ml, Etidote 128 µg/ml+ZB 256 µg/ml, Etidote 128 µg/ml+ZB 64 µg/ml, Etidote 512 µg/ml+ZB 32 µg/ml, Etidote 256 µg/ml+ZB 32 µg/ml, Etidote It showed Additive effect (>0.5 and <1) at 128 µg/ml+ZB 32 µg/ml and Etidote 64 µg/ml+ZB 32 µg/ml doses. Our other concentrations were found to be ineffective in terms of FIC value.

In **Figure 1C**, ZB 32 µg/ml+SPM 128 µg/ml, ZB 64 µg/ml+SPM 64 µg/ml, ZB 32 µg/ml+SPM 64 µg/ml, ZB 64 µg/ml+SPM 32 µg/ml and ZB 32 µg/ml+SPM 32 µg/ml Synergistic effect ≤ 0.5: detected at concentrations. If additive effect (>0.5 and <1); ZB 64 µg/ml+SPM 1024 µg/ml, ZB 512 µg/ml+SPM 512 µg/ml, ZB 64 µg/ml+SPM 256 µg/ml, ZB 32 µg/ml+SPM 256 µg/ml, ZB 128 µg/ml+SPM 128 µg/ml, ZB 64 µg/ml+SPM 128 µg/ml, ZB 256 µg/ml+SPM 64 µg/ml, ZB 128 µg/ml+SPM 64 µg/ml, ZB 256 µg/ml+SPM 32 µg/ml, ZB 128 µg/ml+SPM 32 µg/ml was determined at rates. Other concentrations were found to be ineffective.

The optical density (570/OD) results of the combinations made with the microdilution plate method are summarized in **Figure 2**. In **Figure 2A**, The highest effect on biofilm formation was detected at 32 µg/ml Etidote+256 µg/ml SPM. In **Figure 2B**, The highest effect on biofilm formation was detected at the concentration of Etidote 128 µg/ml+ZB 1024 µg/ml. In **Figure 2C**, The highest effect on biofilm was detected at the concentration of ZB 1024 µg/ml+SPM 256 µg/ml.

MTT Assay

We evaluated the toxicological effects of ZB, SPM and etidote on the HepG2 cell line. In our study, according to our results, ZB+Etidote effectively protected HepG2 cells against *Enterococcus faecalis* ($p>0.05$). Additionally, SPM+ZB protect cell viability but near to 22% cell lost vas evaluated. In addition, SPM+ZB did not protect cell viability ($p<0.05$) (**Figure 3**).

Immunohistochemical Evaluation

In H2A. X and nuclear DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the predominant form of free radical-induced oxidative lesions. In line with the previous findings, H2A. X and 8-OHdG fluorescent signals were not observed in the control group. Several 8-OHdG fluorescence-cent signals were observed in the SPM+Etidote group, moderate signals were detected in the SPM+ZB group, and light signals were easily observed in the ZB+Etidote group (**Table 1**) (**Figure 4**).

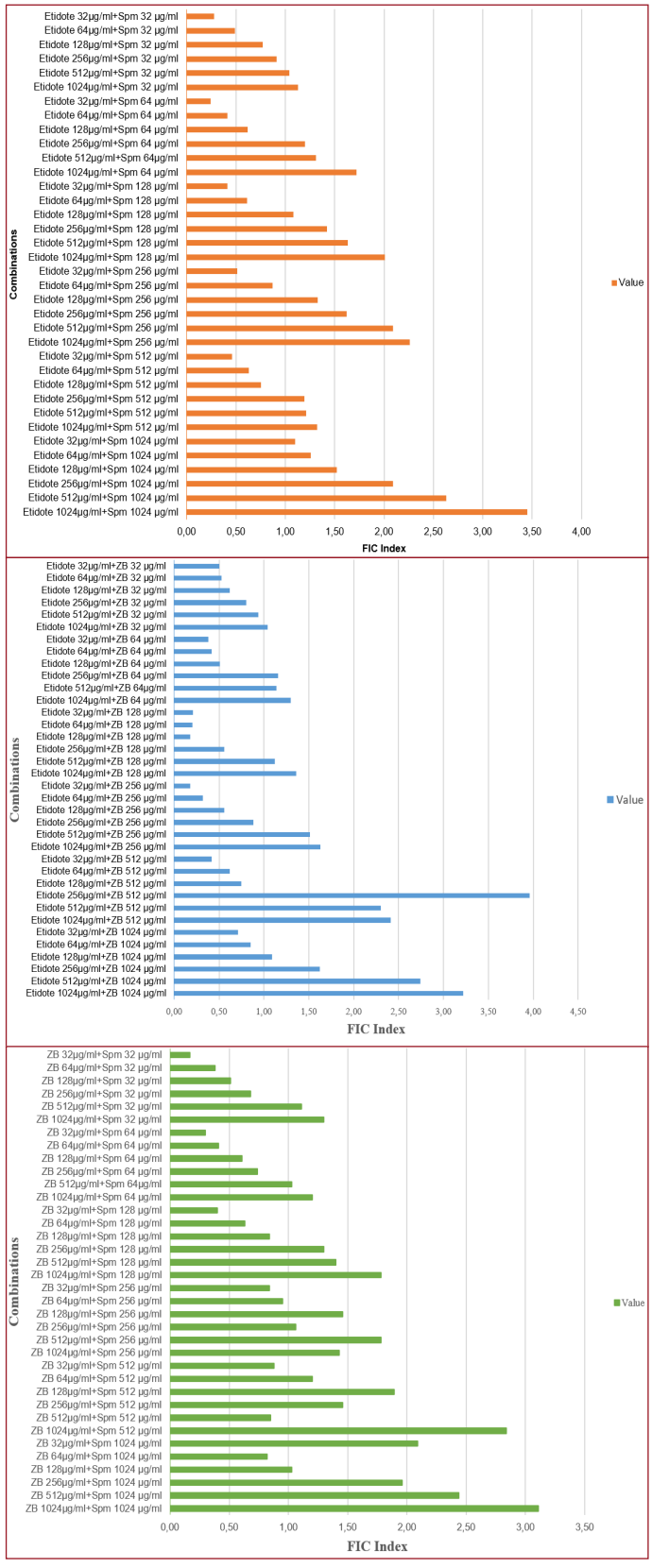

Figure 1. Boron compound FIC index results. A) Etidote+SPM combination FIC index, B) Etidote+ZB combination fix index, C) ZB+SPM combination fix index. Value ranges of boron combinations corresponding to Σ FIC index ≤ 0.5: synergy, >0.5 and <1: additive and ≥1 and 4 ≤: ineffective (indifference).

Table 1. Statistical analysis of immunofluorescent staining results.

	8-OHdG	H2A.X
Control	20.45±5.38[a]	18.68±5.37[a]
SPM+Etidote	61.36±5.91[b]	59.29±3.27[b]
SPM+ZB	39.84±5.61[c]	33.28±3.88[c]
ZB+Etidote	31.18±4.62[c]	29.75±2.59[c]

*a, b, c: different letters in the same column were considered statistically significant differences. ($p<0.05$)

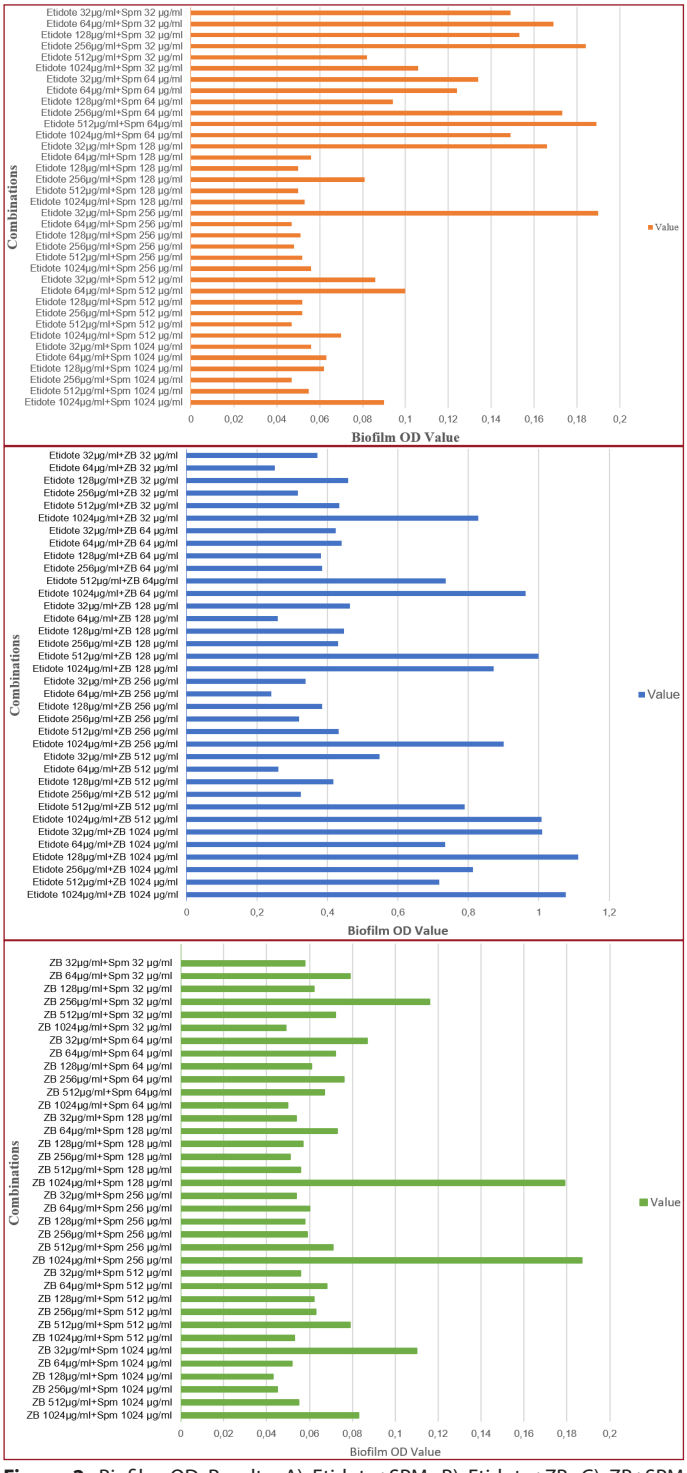

Figure 2. Biofilm OD Results. A) Etidote+SPM, B) Etidote+ZB, C) ZB+SPM biofilm OD values. The minimum and maximum OD values of Etidote+SPM, Etidote+ZB, and ZB+SPM biofilms ranged at 570 OD.

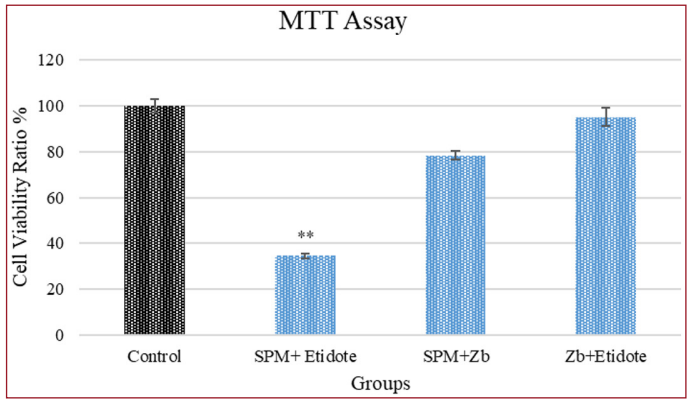

Figure 3. MTT assay results for the HepG2 cell line, control group (received only medium), *Enterococcus faecalis* bacteria cocultured for 24 h with SPM 62,5 µg/ml+Etidote 125 µg/ml, SPM 62,5 µg/ml+ZB 31,25 µg/ml and ZB 31,25 µg/ml+Etidote 125 µg/ml. (*$p<0.05$ compared to the control group).

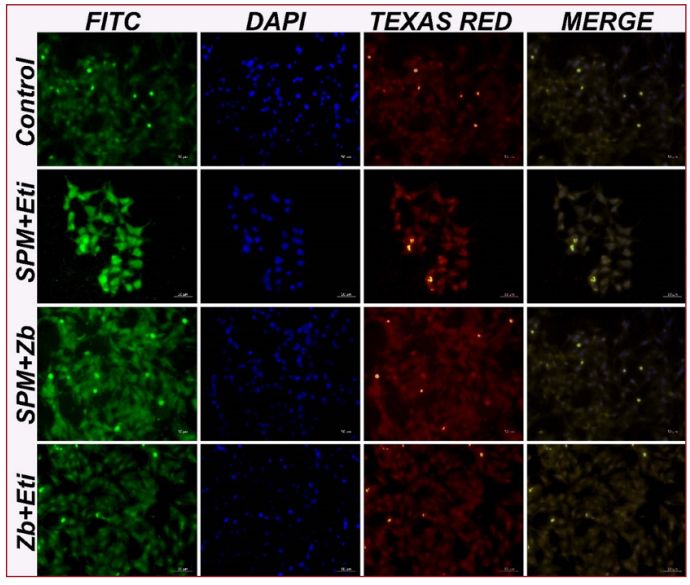

Figure 4. Cell lines, 8-OHdG expression (FITC) and H2A. X expression (Texas Red), IF, Bar: 50 µm.

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. For statistical analyses, all calculations were performed using SPSS 20 software, and $p<0.05$ was considered to be a significant difference in all tests.

DISCUSSION

Enterococcus faecalis, which causes significant infection after liver transplantation, is difficult to treat due to its antimicrobial resistance and biofilm mechanism. We aimed to evaluate the synergistic effect of boron compounds and their effect on biofilms in an infection model created with *Enterococcus faecalis* 29212 on the HepG2 liver cell line to facilitate treatment with alternative treatment methods. *E. faecalis* is a factor that has started to pose a danger in hospital infections.

Resistance to ampicillin, which is routinely used, has been reported at a rate of 1.8% recently. The use of vancomycin is increasing to prevent this resistance from spreading and spreading to community-acquired infections.[17,18] However, hospital-acquired infections (HAIs) caused by vancomycin-resistant enterococci (VREs) are emerging as an additional burden on patients and healthcare systems globally, leaving limited therapeutic options.[19,20] The presence of its core detection system contributes to the spread of this resistance. In this respect, an alternative strategy, such as the degradation of the biofilm layer, is among the methods used to combat multidrug-resistant bacteria.[21] Antimicrobial studies targeting Fsr and cytolysin quorum-sensing systems have been carried out in vitro and in vivo, but it has been reported that more information is still needed about the mechanisms. [22-25] Investigation of synergistic effects with antimicrobial photodynamic therapy (aPDT) models in biofilm eradication[26], application of antimicrobials depending on antimicrobial lock therapy (ALT) and MIC values[27], and methods applied using antimicrobial peptides (AMPs).[28] Prevention of biofilm layer with electrical methods It is promising that resistance to antimicrobials does not develop and that it is not toxic. [29] Medical devices associated with biofilm formation are among other alternative searches in studies conducted with the method of coating with antibiofilm layers to prevent microorganisms from adhering to surfaces.[30] Boron, one of the alternative molecules in all these searches, has recently taken its place in the literature.[31,32] In studies on boron, information on cytotoxic activities in cell culture is scarce.[33] In a study using antibiofilm analysis, boric acid and etidote MICs were found to be between 0.77-3.09 mg/ml and 0.644-10.312 mg/ml, respectively.[34] In our study, Etidote 32 µg/ml+Spm 256 µg/ml had the highest effect on biofilms, Etidote 128 µg/ml+ZB 1024 µg/ml had the highest effect on biofilms, and ZB 1024 µg/ml+SPM 256 µg/ml had the highest effect on biofilms. detected at the highest rate. This effect, determined at lower concentrations, is promising. In another study, in which the cytotoxicity of tetra acetyl ethylen diamine-sodium perborate and sodium hypochlorite was compared, the cytotoxicity of the substances used in the study was examined at doses ranging from 0.0025% to 0.5%. In our study, it was determined that ZB+Etidote preserved a vitality rate of 95%. Similar results were found in the IF experiment, and DNA damage was shown to be minimal. However, our findings showed that the SPM+ZB group did not provide any protection against bacteria in the HepG2 cell line in the cellular environment.

CONCLUSION

The study is promising in terms of containing new information in terms of cell culture content, immunohistochemical analysis results and FIC concentrations. However, more comprehensive in vivo studies and determination of their effects at the molecular level are needed to determine the activities of these alternative compounds from living things.

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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