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چکیده

مقدمه: فرسودگی زیستی میراث فرهنگی پدیده‌ای جهانی است که ارتباط نزدیکی با حضور ماکروارگانیسم و میکروارگانیسم‌هایی مانند باکتری‌ها، آرکی‌ها، قارچ‌ها و گلسنگ‌ها و متابولیت‌های آنها دارد. چنین جوامعی به آسیب فیزیکی و شیمیایی و همچنین تغییر زیبایی در آثار فرهنگی منجر می‌شوند. برای کنترل فرسودگی زیستی ناشی از رشد میکروارگانیسم‌های میراث فرهنگی، از مواد ضدعفونی کنندهٔ مختلف استفاده شده است. این مطالعه بر استفادهٔ مؤثرتر از ضدعفونی کننده‌های شیمیایی متداول در حوزهٔ حفاظت از میراث فرهنگی متمرکز شده و روشی برای کاهش میزان مصرفی میزان ضدعفونی کننده‌ها در کنترل رشد و حذف میکروارگانیسم‌ها پیشنهاد کرده است که به کاهش آسیب‌های زیست‌محیطی منجر می‌شود.

مواد و روش‌ها: در این مقاله اثرات ضدمیکروبی ضدعفونی کننده‌های متداول ازجمله کلرید بنزالکونیوم، ایزوتیازولینون، فرمالین، گلوتارآلدئید و پودر ضدعفونی کنندهٔ مبتنی بر اکسیژن علیه باکتری‌ها و قارچ‌های جداشده از سنگ آرامگاه کوروش بزرگ با روش میکروبراث بررسی شدند. سپس اثر ترکیبی ضدعفونی کننده‌ها با استفاده از روش چکربورد مطالعه شد.

نتایج: نتایج آزمون‌ها نشان دادند گلوتارآلدئید و پودر ضدعفونی کنندهٔ مبتنی بر اکسیژن، MIC بیشتری نسبت به سایر ضدعفونی کننده‌ها یعنی کلرید بنزالکونیوم، ایزوتیازولینون و فرمالین داشتند.

بحث و نتیجه‌گیری: نتایج نشان دادند تمام ضدعفونی کننده‌های انتخاب‌شده در این مطالعه، اثرات ضدمیکروبی قابل قبولی بر میکروارگانیسم‌های جداشده از سنگ آرامگاه کوروش داشتند و ترکیب ضدعفونی کننده‌ها موجب شد مقدار کمتری از این مواد شیمیایی استفاده شود که به لحاظ زیست‌محیطی بسیار حائز اهمیت است.

واژه‌های کلیدی: اثرات ضدمیکروبی، فرسودگی زیستی، حفاظت، جوامع میکروبی، میراث فرهنگی

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A New Approach to Eliminate the Microorganisms Involved in the Deterioration of Stony Cultural Heritage by Biocides

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Abstract

Introduction: The biodeterioration of cultural heritage is a worldwide phenomenon. It is closely linked to the presence of microorganisms such as bacteria, archaea, fungi, lichens, and their metabolites. Microbial colonization leads to physical and chemical damages as well as aesthetic changes of ancient objects. Biocides have been used for all kinds of cultural heritage material to control microbial growth as well as its deterioration. The present study focuses on applying current chemical biocides to improve conservation strategies of the cultural heritage and develops a way to reduce consumption of biocides to prevent the growth of microorganisms and reduce environmental biological damages.

Materials and Methods: In this study, the antimicrobial effects of commonly used biocides including benzalkonium chloride, isothiazolinone, formalin, glutaraldehyde, and active oxygen-based disinfectant powder were examined against bacteria and fungi isolated from Cyrus the Great tombstone by microdilution broth methods and the combined effect of biocides was studied by the microdilution chequerboard method.

Results: Microdilution tests showed that glutaraldehyde and active oxygen-based disinfectant powder had higher MIC values than other biocides, benzalkonium chloride, isothiazolinone, formalin. On the other hand, other biocides were more effective against the tested bacteria.

Discussion and Conclusion: The results of the study showed that all selected biocides had acceptable antimicrobial effects on microorganisms isolated from Cyrus the Great tombstone alone. When biocides were combined, they had a better antimicrobial effect resulting in less use of these materials.

Key words: Antimicrobial Effects, Biodeterioration, Conservation, Cultural Heritage, Microbial Communitie

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Introduction

One of the major causes of deterioration of cultural heritages and artworks is biodeterioration caused by microorganisms. Biodeterioration can be described as "any undesirable change in a material brought about by the vital activities of organisms" (1). The metabolites of microorganisms are responsible for the deterioration of the underlying substrate and may induce physical weakening and discoloration of the stone (2). To eliminate the microorganisms and prevent their recurrence for a short time, different kinds of treatments can be applied to deteriorated materials (1). Actions against microbial growth can be divided into four main classes: A) indirect control by modifying environmental conditions; B) mechanical control by removing microorganisms; C) chemical control by using biocides; and D) physical methods by application of high temperatures, radiation, filtration, and drying (3).

For disinfection of microorganism's damage, there is a limited range of physical and chemical methods (4). Liquid biocides and fumigation with gases are used as chemical treatments. The European Union's Biocidal Products Directive (BPD) limited the choice of biocides because only a small number of agents have been tested regarding their compatibility with historic materials, and few studies have been performed on their long-term effects such as color alterations, degradation of treatments, and appearance of an object after restoration (5). Dresler et al. examined two commercial biocides, New Des 50 and Biotin T used in restoration works (13). Frequently used Biocides in restoration are formaldehyde releasers, quaternary ammonium compounds, and isothiazolinone (5, 6). A wide variety of traditional chemical biocides have some advantages such as accessibility in markets, being cheap, and generally easy to apply (2, 3). They are effective against a broad range of microorganisms. Furthermore, they are applicable in distant areas. However, traditional chemical biocides have some drawbacks including toxicity for operators and environments. Moreover, the low long-term effectiveness, promotion of biocide-resistant communities, possible modification of biofilm structures favoring the growth of more harmful biodeteriogens, and the repeated use may damage the heritage material (7, 8). Despite the accepted short-term biocides efficiency, the removal of the microbial community may cause a new succession of microorganisms, which may be more destructive than the old microbial populations (9). Against microbial recolonization, some surveys have suggested that the combined application of different kinds of biocides is more effective than using only one biocide (5, 10). As a new disinfectant, the active oxygen-based disinfectant powder was formulated by Padideh Shimi Gharb CO used for disinfecting and cleaning medical devices and other surfaces in the industry. This powder has strong antibacterial and antifungal properties and decomposes to water and oxygen and safe for the environment. Therefore, in the present study, the authors decided to evaluate the biocide efficiency against cultural heritage isolates as a new application.

First, the antimicrobial effect of common biocides used in stone cultural heritage restoration was tested against bacteria and fungi isolated from Cyrus the Great tombstone. Then, in order to reduce biocide consumption and environmental pollution, the combined application of biocides was examined, and finally, a new active oxygen-based disinfectant powder was formulated and its antimicrobial effects were evaluated, respectively.

Materials and Methods

Preparation of Biocides: In this study, various disinfectants including benzalkonium chloride, formalin, glutaraldehyde, isothiazolinone, and active oxygen-based disinfectant powder were used. The stock solution of benzalkonium chloride was prepared under the brand names Acticide BAC 80 (concentration 80% in H_2O), Acticide BAC 50 (concentration 50% in H_2O), with a concentration of 1000 µg/mL. The stock solution of isothiazolinone was prepared under the brand name POLICIDE BBN, with a concentration of 1000 µg/mL. The stock solution of glutaraldehyde was prepared at a concentration of 40,000 µg/mL. Formalin is the saturated solution of formaldehyde in water and formalin solution 100% is equivalent to 37%–40% formaldehyde, which was prepared at a concentration of 20000 µg/mL. The stock active oxygen-based disinfectant powder under the brand name PSGPRO (PSG, IRAN) with a concentration of 5000 µg/mL was prepared (4, 5).

Bacterial and Fungal Strains: All microorganisms used in this investigation were isolated from the Cyrus the Great tombstone in Iran and identified using the classic and molecular methods. Isolated bacteria including *Bacillus cereus*, *Arthrobacter pascens*, *Arthrobacter oryzae*, *Bacillus samanii*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus endophyticus*, *Massilia timonae*, *Bacillus simplex*, *Bacillus firmus* were kept on BR2 agar and stored at 4 ° C. Fungal isolates including *Fusarium*, *Cladosporium*, and *Candida albicans* were kept on potato dextrose agar (PDA) and stored at 4 ° C (6). All isolates were deposited at the microorganism bank of Dr. Sepehr Research Laboratories, Alzahra University, Tehran (6, 7).

Determination of the Antibacterial Activity of Biocides: The Minimum Inhibition Concentration (MIC) of biocides was assayed according to the CLSI (Clinical and Laboratory Normal Institute) by the microdilution method. Initially, 100 µL of Muller Hinton Broth (MHB) was added to all the first nine wells. Then, 100 µL of biocide (1 mg/mL) was added to the first well of the microtiter plate. After that, the dilution of biocide was carried out till the ninth well. At the final step, 100 µL of bacterial suspension with concentrations equal to 5×10^6 CFU/mL was added to the first nine wells. The three wells at the end of the microtiter plate were used as control of biocide, culture media, and bacteria, respectively. The microtiter plates were incubated for 18-24h at 37 ° C. Then, 100 µL of each serial dilution was taken from each well and spread on the nutrient agar to obtain the Minimum Bactericidal Concentration **(**MBC). Its lowest concentration that created three or fewer colonies (i.e. 99% of the inoculum was killed) was identified as the MBC (8).

Determination of the Antifungal Activity of Biocides: Antifungal tests were carried out using the M38-A (NCCLS, 2002) micro broth dilution method with slight modification (9). The fungal cells were obtained by growing the isolates on PDA for 5 days at 28±2°C. Then, fungal conidia were collected by using 3-4 mL of sterile saline solution, by gently scraping off the medium surface. The suspension was spectrophotometrically adjusted to 0.05- 0.1 of OD_{530}, which is equal to 10^5 CFU/mL. Initially, 100 µL of sabouraud dextrose broth (SDB) was added to all the first nine wells. Then, 100 µL of biocide (1 mg/mL) was added to the first well of the microtiter plate. After that, the dilution of biocide was carried out till the ninth well. At the final step, 100 µL of fungal suspension with concentrations equal to 10^5 CFU/mL was added to the first nine wells. The lowest concentration of biocide with no visible growth development occurred after 72 h of the incubation at 28 ° C was considered as the MIC end-point.

Then, 100 µL of each serial dilution was taken from each well and spread on sabouraud dextrose agar (SDA) to obtain the Minimum Fungicidal Concentration (MFC). Its lowest concentration that created three or fewer colonies (i.e. 99% of the inoculum was killed) was considered as the MFC (10).

Determination of Fractional Inhibitory Concentration (FIC) and FIC Index (FICI): The combined effect of disinfectants was evaluated by the microdilution chequerboard method. The FIC value may vary from those of in *in vivo* assays because of positive and negative interactions. Assays were performed on 96-well polypropylene microtiter plates based on disinfectant MIC0 values which were previously obtained. For this reason, five concentrations of disinfectants were prepared (MIC0/4, MIC0/2, MIC0, 2 MIC0, and 4 MIC0). Then, 50 µL of each disinfectant dilution was added on the x-axis across the chequerboard plate, while other disinfectant dilutions were dispensed on the y-axis to obtain the final concentrations of the fractional inhibitory concentration (FIC) with MIC0/4, MIC0/2, MIC0, 2 MIC0, and 4 MIC0 for each disinfectant (11).

Next, 100 µL of bacterial suspension equal to 0.5 McFarland standard turbidity was added in each well, and in order to evaluate the FIC of disinfectants against fungal strains 100 µL of fungal conidia suspension with concentrations equal to 10^5 CFU/mL was added in each well, except the negative control, which was added with 100µL of sterile MHB for bacteria and 100 µL of sterile SDB for fungi. The microplates were incubated at 37 ° C for 24 h for bacterial strains and 72 h at 28 ° C for fungal strains. Then, FICI values were calculated using the following formula (11, 12):

FICI= FICA + FICB Where FICA = MICA in combination/MICA alone and FICB= MICB in combination/MICB alone

Results

Results of Antibacterial Activity of Biocides: The MIC of Acticide BAC 50, Acticide BAC80, and POLICIDE BBN, glutaraldehyde, formalin, and active oxygen-based disinfectant powder has been assessed against bacteria isolated from Cyrus the Great tombstone. In this step, several concentrations of biocides were tested, and each was in duplicate. In figures 1 and 2, the results of microdilution tests showed that glutaraldehyde and active oxygen-based disinfectant powder are less effective than other biocides. The active oxygen-based disinfectant powder had the highest MIC value of about 5000 µg/ mL, formalin and benzalkonium chloride had the lowest MIC value of about 750- 1000 µg/ mL. In general, all the isolates were susceptible to biocide treatment by using the microdilution method. The high antimicrobial properties of biocides belonged to BAC, formalin, and isothiazolinone against all the isolates.

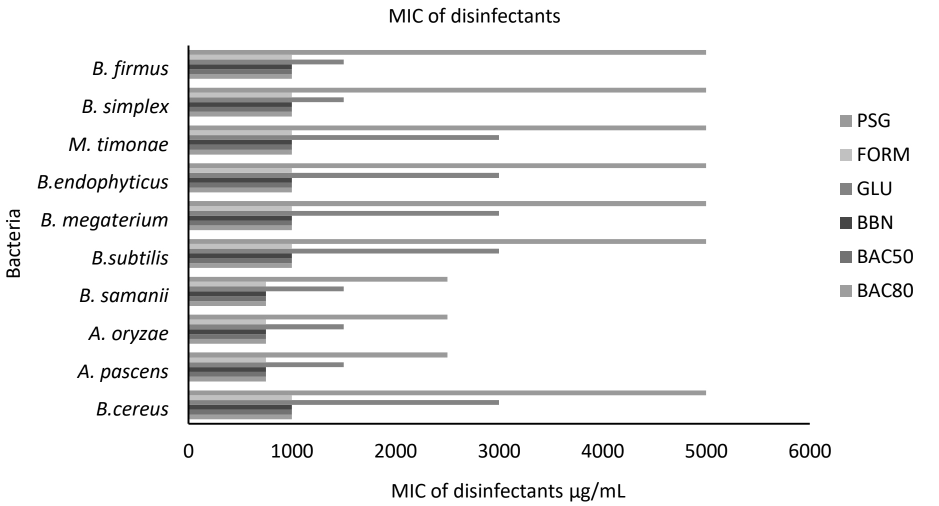

Fig. 1- MIC of Disinfectants against Bacterial Strains
Abbreviations: PSG: active oxygen base disinfectant powder under the brand name PSGPRO, FORM: formalin, GLU: glutaraldehyde, BAC 80: benzalkonium chloride under the brand names Acticide BAC 80 BAC50: benzalkonium chloride under the brand names Acticide BAC 50, BBN: isothiazolinone under the brand name POLICIDE BBN

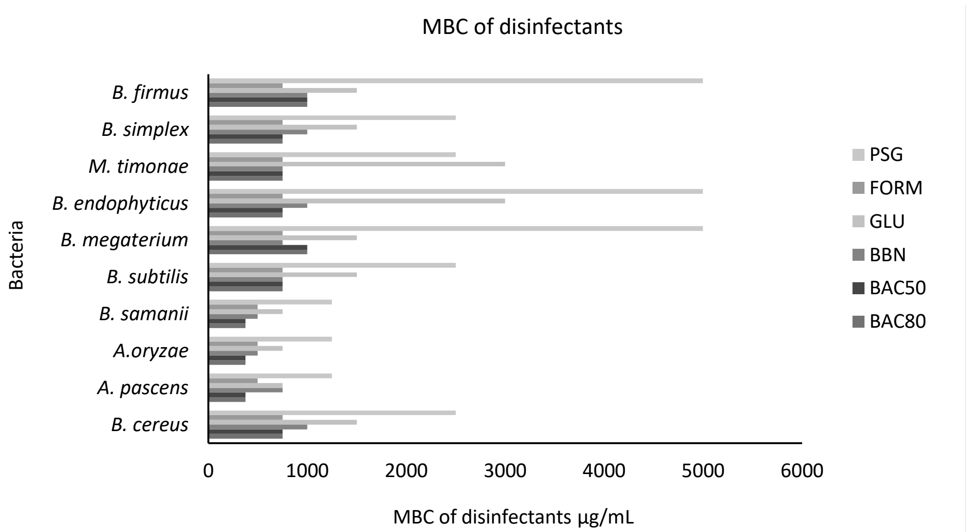

Fig. 2- MBC of Disinfectants against Bacterial Strains
Abbreviations: PSG: the active oxygen-based disinfectant powder under the brand name PSGPRO, FORM: formalin, GLU: glutaraldehyde, BAC 80: benzalkonium chloride under the brand names Acticide BAC 80, BAC50: benzalkonium chloride under the brand names Acticide BAC 50, BBN: isothiazolinone under the brand name POLICIDE BBN

Results of Antifungal Activity of Biocides: Several concentrations of biocides were tested against *Cladosporium*, *Fusarium*, and *Candida albicans*, and each in duplicate. The results of microdilution tests showed that glutaraldehyde and PSG powder are less effective than other biocides. The active oxygen-based disinfectant powder had the highest MIC value about 5000 µg/mL. Formalin and benzalkonium chloride had the lowest MIC value about 750- 1000 µg/ mL.

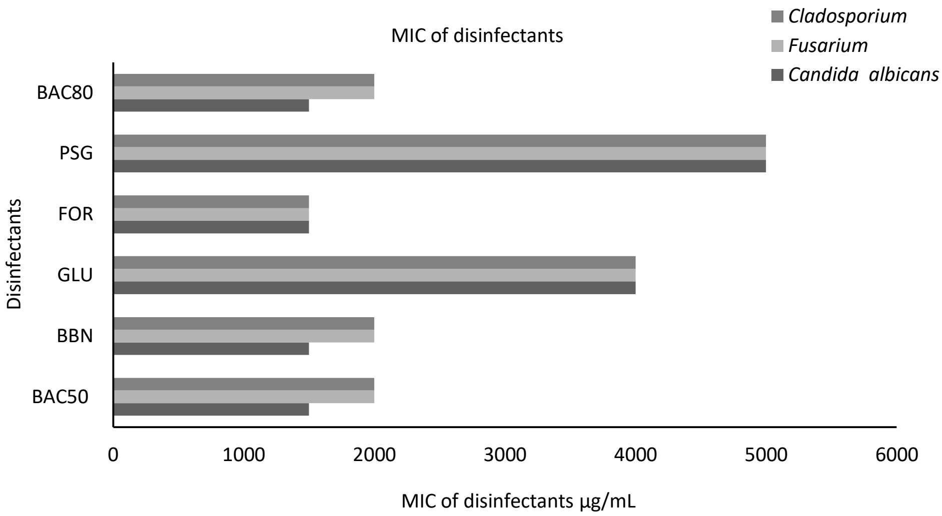

Fig. 3- MFC of disinfectant against fungal strains

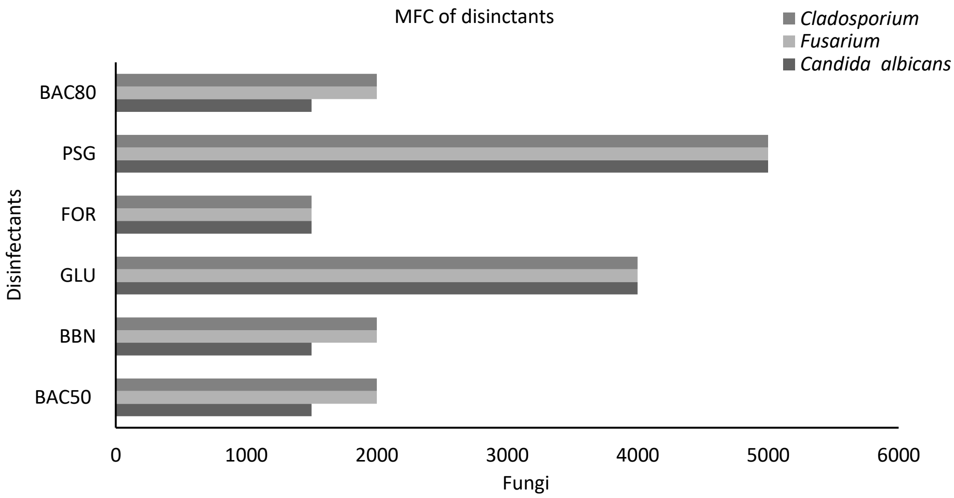

Fig. 4- MFC of disinfectant against fungal strains

Results of FIC and Determination of FICI: The degree of synergy between disinfectants is shown in terms of the Fractional Inhibitory Concentration (FIC). The FIC is the MIC of disinfectant in combination divided by the MIC of disinfectant alone.

For each well, the sum of the FICs (∑ FIC) was calculated with the equation ∑ FIC = FICA + FICB = (CA/MICA) + (CB/MICB), where MICA and MICB are the MICs of disinfectant A and B alone, respectively, and CA and CB are the concentrations of the disinfectants in combination.

The FIC values for each disinfectant were considered from 250 to 2000. Table 1 shows the FIC values for the combination of the following disinfectants: BAC 50 / formalin, BAC50 /glutaraldehyde, BBN / formalin and BBN/ glutaraldehyde against *Bacillus cereus* and *Candida albicans* because they are frequently identified on cultural heritage materials.

A Synergistic effect (SynE) is observed when FICI value ≤ 0.5; an additive effect (AddE) when $0.5 < \text{FICI value} \leq 1$; an Indifferent effect (IndE) when $1 < \text{FICI value} < 2$ and an Antagonistic effect (AntE) when FICI value ≥ 2. The results showed that there are additive and synergistic effects in combination with selected disinfectants. When isothiazolinone and formalin were used in combination, synergistic effects were observed.

Table 1- FIC value of different disinfectant

Microorganisms	FIC BAC 50	FIC FORMALIN	FICI	RESULTS
B. cereus	0/5	0/25	0/75	AddE
C. albicans	0/5	0/5	1	AddE
Microorganisms	FIC BAC 50	FIC glutaraldehyde	FICI	RESULTS
B. cereus	0/5	0/5	1	AddE
C. albicans	0/5	0/5	1	AddE
Microorganisms	FIC BBN	FIC glutaraldehyde	FICI	RESULTS
B. cereus	0/25	0/5	0/75	AddE
C. albicans	0/5	0/5	1	AddE
Microorganisms	FIC BBN	FIC FORMALIN	FICI	RESULTS
B. cereus	0/25	0/25	0/5	SynE
C. albicans	0/5	0/5	1	AddE

Discussion and Conclusion

Physical and chemical damage, as well as aesthetic alteration, are consequences of the microbial deterioration of cultural heritage (14). To remove these microorganisms from the surfaces of cultural heritages, different kinds of techniques have revealed new opportunities for microbiologists and conservators. These techniques include chemical methods, for instance, traditional biocides and nanoparticles; physical methods such as mechanical removal, UV irradiation, and biological methods such as natural molecules with biocidal properties, enzymes, and microorganisms (1, 15).

In this study, we evaluate the efficacy of traditional biocides against bacteria and fungi isolated from the tomb of Cyrus the Great. However traditional chemical biocides have some advantages and drawbacks. Their advantages include:

- Wide varieties of compounds are available on the market.
- They are inexpensive and usually easy to use.
- They have broad-spectrum antimicrobial properties against microorganisms.
- They can be used in remote areas.

Their drawbacks include:

- They are toxic to workers and the environment.
- Their long-term effectiveness is very low.
- They are often not selective against specific microorganisms.
- Microorganisms may become resistant to these biocides.
- Repeated use of chemical disinfectants may damage the heritage material (16).

We observed that quaternary ammonium salts had acceptable antimicrobial properties. Ascaso et al. studied the effects of biocides on microorganisms isolated from carbonate rock of the Jeronimos Monastery. Their results showed that the use of quaternary ammonium salts led to the complete disorganization of microorganisms (17).

Cappitelli et al. examined antimicrobial properties of commonly used biocides such as BAC and isothiazolinone to eradicate the microorganisms from stones. Their results showed that 0.5% was the optimal concentration of isothiazolinone, while our results showed that 0.1% was its optimal concentration (18). However, in both studies, isothiazolinone showed acceptable antimicrobial properties.

Antifungal activity of BAC was reported by Stupar et al. against the fungi isolated from cultural heritage objects. Their results indicated that benzalkonium chloride displayed the strongest antifungal activity among the used biocides. In our study, benzalkonium chloride also displayed strong antifungal properties, which was similar to

Stupar et al.'s reports (10).

Koziróg et al. studied the efficiency of microbicides for the disinfection and protection of historical wooden surfaces. They evaluated glutaraldehyde, isothiazolinone, and quaternary ammonium compound against bacteria and fungi isolated from historical wood. Their results indicated that these biocides were effective against microorganisms colonizing on the wood surface. Our results were similar to Koziróg's study concerning the efficacy of mentioned biocide (2).

It is important to mention when the same biocides are repeatedly used, microorganisms may become resistant to these biocides (19). To avoid increasing the biocidal resistance of microorganisms, the replacement of biocides is often recommended (5).

Combination therapy is mainly used because of broadening the antibacterial spectrum, synergy, and mitigating the emergence of resistance (12).

Consequently, we decided to evaluate the combined effect of biocides against microorganisms isolated from the stony cultural heritage of Pasargadae. The results of this study confirmed the hypothesis and the combined disinfectants had a synergistic impact. The use of combination doses reduced the biocide consumption which is friendlier for the environment.

The active oxygen-based disinfectant powder is used for disinfecting and cleaning medical devices as well as all types of surfaces. This powder is suitable to use in risk areas, where a user-friendly surface disinfectant with a broad range of effectiveness is necessary. Because of the mentioned reasons, we decided to examine the antimicrobial effects of this disinfectant in the field of cultural heritage for the first time. The results indicated that the MIC value of the active oxygen-based disinfectant powder against the selected strains is higher than other disinfectants. Since the oxygen-based disinfectant powder degrades to water and oxygen, it is recommended to use and is safe for the environment.

It can be concluded that the combined biocides had synergistic impacts. Moreover, the use of combined biocides reduced the biocide concentration, which is more appropriate for the environment. In this research, the mentioned disinfectants were selected based on their applications and compatibility with historical objects. In the case of a new active oxygen-based disinfectant, it is recommended to evaluate its compatibility with the stone of Cyrus the Great tomb in future studies. Further investigation should be carried out to suggest the best combination of commercial biocides with less concentration as well as less environmental destruction.

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