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ORIGINAL ARTICLE

Comparative examination of in vitro methods to evaluate the antimicrobial activity of biomaterials

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Abstract

One of the basic building blocks of biomaterials is antimicrobial activity. It is an unacceptable misconception that this property cannot be determined due to incorrect methods and faulty microorganism selection. This study covers the determination of the most appropriate microbial activity test according to the type (metal, polymer, ceramic coating) and chemical form (liquid, solid) of the biomaterial by comparing methods. In our study, there are four biomaterial groups: Metal (titanium, zinc, iron), metal alloy (Ti6al4V, iron manganese), Polymer (polymethylmethacrylate, polycaprolactone, polylactic acid and polyhydroxy butyrate), Composite polymer (polyhydroxybutyrate-valerate), Ceramic and Additives (hydroxyapatite, titanium dioxide, zinc oxide) and Composite material (zinc titanium oxide) with different amounts of Zinc). Microorganisms in the experimental studies included gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Staphylococcus epidermidis*), gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi* and a fungus (*Candida albicans*). Antimicrobial assays are performed by 1-Diffusion methods (Agar disk diffusion method), 2- Antimicrobial gradient method (Etest), 3- Serial dilution, 4-Time kill test (time kill curve) and 5- Colony counting method. The possible antimicrobial activities of PHB, C-PLA and PHB-V polymers and the superior antimicrobial activity results of zinc and metal-based composite biomaterials were clearly determined by utilizing the correlations of multiple test methods. Colony counting and time-kill tests were linearly correlated with other test methods, mainly depending on the solubility of the biomaterials. If researchers know the structural characteristics of the biomaterial they have, this study explains which microorganisms and which test methods will lead to the correct result. The most different and most necessary motivator in our study is the need to recognize that the compatibility between the antimicrobial activity studies of biomaterials is as perfect and single-solution oriented as the key-lock compatibility. A simple template for basic antimicrobial activity testing methods for metal, ceramic, polymer and composite biomaterials.

Keywords Antimicrobial tets · Biomaterials · Biopolymers · Composite materials · Method development · Time kill methods

1 Introduction

Biomaterials are natural or synthetic substances and mixtures that provide support based on the structural function of the cells in artificial tissues or functional organs that are integrated and in contact with the fluids in the body, including blood and tissue fluids used for treatment or diagnosis, and do not have any adverse effect on the living being [1]. In addition to structural properties such as biocompatibility, corrosion resistance, bioactivity, wear resistance, and osteointegration, antimicrobial activity in all biomaterials explicitly used for treatment purposes is one of the most sought characteristics [2].

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We can define an antimicrobial agent as a substance that kills microorganisms such as bacteria, yeasts, or molds or suppresses/reduces their growth. Everything that affects microbes is considered 'antimicrobial', but the term antimicrobial is not a benchmark for performance. The effectiveness of the antimicrobial agent depends on various parameters. Antibacterial, antifungal, and antiviral activity experiments are carried out to evaluate substances' antimicrobial activities and optimize their concentration [3].

There are various efficacy tests used to assess microbial activity. Therefore, more attention should be paid to evaluating and selecting antimicrobial activity methods Some techniques have been standardized by CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee for Antimicrobial Susceptibility Testing). However, when testing substances, some modification of standardized protocols is often in demand. It can be said that making minor methodological adaptations to standardized protocols can be an excellent solution to ensure the correct experimental approach and allow other researchers to compare the results [4].

Various in vitro methods are used to determine the antimicrobial activities of substances and materials to determine the desired result. To achieve the most stable and expected result in experimental studies, choosing the most appropriate one for the experiment from the in vitro antimicrobial activity test methods is one of the essential situations within the scope of the studyIn selecting the method, the number, quantity, solubility of the substance/substances, and the type, property, and density of the microorganism to be used are also issues to be considered. In addition to the reliable and reproducible results, preference should be given to the method that can be completed in the shortest time with the least effort and is the most economically modest [3].

As there are compelling factors in the selection of antimicrobial test methods, the advantages and differences between each other should be carefully examined. Various bioassays, such as disc diffusion, well diffusion, and liquid or agar dilution, are well-known and widely used. Methods for in vitro evaluating antimicrobial activity: (A review") As suggested, while these guidelines provide a uniform testing procedure that is practical in most clinical microbiology laboratories, developing such methodological standards does not guarantee the clinical relevance of such tests. However, it allows bioassay to be performed with a standardized approach to assess the clinical extent of the results [4].

Nowadays, many researchers have focused on the search for plant and microbial extracts, essential oils, pure secondary metabolites and newly synthesized molecules as potential antimicrobial agents. In addition to all these studies, microbial activity of biomaterials has also been frequently included in the literature. However, when we review published papers on the microbial effect of materials, it is often difficult to compare results due to different non-standardized approaches (strain preparation techniques, inoculum size, growth media). Especially since the evaluation of the microbial activity of biomaterials is within the scope of multidisciplinary studies, sometimes incorrect methods are chosen or different approaches to analyze the results emerge. A better understanding of the methods available for screening and measuring the antimicrobial effect of a biomaterial is a very important factor with a profound impact.

2 Materıal and methods

2.1 Material

Biomaterials: Various biomaterials from 4 different biomaterials were used in experimental studies: The biomaterial groups included in the research and the types of materials within these groups are:

- Metal—Metal Alloy Biomaterials: Ti6Al4V, Zn, FeMn (Iron manganese), FeMnZn (Iron Manganese oxide)
- Polymer, Copolymer Biomaterials: PHB (Polyhydroxybutyrate), PLA (Resinex C Polylactic Acid), PHB-PLA (Polyhydroxybutyrate- Polylactic Acid), PCL (Polycaprolactone), PBHV (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), PMMA (Poly(methyl methacrylate).
- Composite Biomaterials: $ZnTiO_2$ (ZincTitanium oxide)
- Ceramic Biomaterials and Additives: HA, TiO_2, and ZnO (Hydroxyapatite, Titanium oxide and Zinc oxide).

Microorganisms Gram has positive characteristics; Gram-negative properties when using RSKK 02.001 *Staphylococcus aureus,* RSKK 96086 *Bacillus subtilis*, RSKK 08.021 *Staphylococcus epidermidis,* and the human pathogen *Enterococcus faecalis* found in our laboratory; ATCC 25922 *Escherichia coli,* RSKK 703 *Klebsiella pneumoniae,* RSKK 26I *Pseudomonas aeruginosa,* RSKK 1107 *Salmonella typhi* and the fungi species RSKK 35 *Candida albicans*. The mentioned RSSK strains were commercially procured from the Reference Microbiology Laboratory / Refik Saydam Culture Collection within the scope of the Public Health Laboratory of the Republic of Turkey.

Antibiotic discs In this study, there used four types of antimicrobial agents in our laboratory as Bioanalayse brand to control the controlled use and effectiveness of test microorganisms. Tetracycline: *S. typhi* and *S. epidermidis* [5, 6]. Streptomycin: *B. subtilis*, *E. coli,* and *P. aeruginosa* [5, 7, 8]. Penicillin: *E. faecalis* and *K. Pneumoniae* [8–10]. Ampicillin + sulbactam: *S. aureus* [5]. Uniderm Farmaceutici- Candinet: *C. albicans* (impregnated in filter papers) [11].

2.2 Resuscitation of test microorganisms and density optimization

Table 1 shows the culture medium, microbial inoculum size, and incubation conditions for antimicrobial susceptibility test methods, as recommended by CLSI about practical strategies. The following guidelines were observed in the disk diffusion and E test methods [10, 12], the liquid microdilution method for the determination of the minimum inhibition concentration [12, 13] the Time-kill method [14], and the plaque counting methods [15]. The steps required for optimization were carried out in order: Growth of microorganisms after incubation and determination of microorganism density (With Thoma slide) [11].

2.3 Positive and negative control choices in antimicrobial activity methods

The more accurately the control groups are selected and applied, the better the experimental results are kept away from external influences, the optimization of the experiment is ensured, and the interpretation of the results is facilitated. Table 2 contains specially selected positive and negative control groups specific to all methods in our experimental methodology.

2.4 Methods of antimicrobial activity

Within the scope of this study, a detailed evaluation was made about the advantages and limitations of microbial activity tests for diffusion methods (Agar disk diffusion method, antimicrobial gradient method (Etest), Seri dilution, time-killing test (time killing curve), plaque counting method methods.

2.4.1 Agar disc diffusion method

In the agar disc diffusion method, metal–metal alloys, polymers, composites, and ceramic biomaterials were tested with all test microorganisms (Before the experiment, bacterial strains were incubated for 24 h on 4.5 mL of TSB medium at 37 °C until they reached a concentration of 10^8 CFU/mL. Under the same conditions, *C. albicans* were incubated for 24 h up to a concentration of 10^6 CFU/mL (Table 1) [9, 10]. In addition to; since the three types of materials in the ceramic biomaterial group are in powder form, all of them were pressed in special press molds with a diameter of 1 cm in a high-pressure pressing device [11].

Metal-to-Metal Alloys – Composites – Ceramics All materials are sterilized under UV light for 30 min, with each face for 15 min. After sterilization, the amount determined by proportioning the standard value (10^8 CFU/mL = 20 µl) of microorganisms brought to specific standards was performed on the TSA trees. Within 15 min of sowing, the samples were placed 1,5 cm away from each other and the edges of the petri dish. In the middle of the Petri dishes, antibiotic discs suitable for the cultivated strain of microorganisms were placed.

Polymers – Copolymers a section in the agar disc diffusion experiment of polymers and copolymers distinguishes them from other biomaterials. This section is the stage of preparing polymers and copolymers for experimentation. All

Table 1 Antimicrobial susceptibility testing methods recommended by CLSI [4]

	Microorganism	Inoculum Density CFU/mL	Incubation Temperature °C	Incubation Period
Agar Disc Diffusion	*Bacteria*	$(1–2)\times10^8$	35 ± 2	16–18
	Fungi	$(1–5)\times10^6$	35 ± 2	*20–24*
MIC	*Bacteria*	1×10^5	35 ± 2	*20*
	Fungi	$(0.5–2.5)\times10^3$	*35*	*46–50*
E Test	*Bacteria / Fungi*	$(1–2)\times10^8$	35 ± 2	*16–18*
Time-Kill	*Bacteria / Fungi*	1×10^5	35 ± 2	*0–3-6–18-24*
Plaque Counting	*Bacteria / Fungi*	$(1–5)\times10^6$	35 ± 2	*16–24*

Table 2 Positive and negative control groups in antimicrobial activity methods [11]

Methods	Positive Control	Negative Control
Agar Disc Diffusion	Antibiotic Discs	Blank Filter Paper Discs
E Test	Antibiotic Discs	Blank Filter Paper Discs
MIC and Time-Kill	Microorganism + Biomaterial solvent in the medium	Only microorganisms in the medium
Plaque Counting	Culture with antibiotics	Only microorganisms in the medium

polymers and copolymers are first weighed on a 25-µg precision balance at this stage. All polymers and copolymers are then dissolved in 250 µl of chloroform, subject to a 1:1 ratio.

2.4.2 Determination of minimum inhibition concentration (MIC)

These methods are the E-test method and the spectrophotometric measurement method. Since these methods are included in the quantitative method groups, they give effective and clear results for the minimum inhibition concentration [16].

E-test The E-test method in our study was carried out only in the working part with polymers-copolymers according to the dissolution of the material (Table 3). Microorganism density determination was performed because of 24-h incubation of all test microorganism strains 10^8 CFU/mL used before the study (Table 1).

Spectrophotometric measurement method The serial dilution method is the main progress in making spectrophotometric measurements and comparative analysis. The serial dilution method is the observational examination of the substance/material with the control groups due to the 24th-hour incubation after determining the concentrations at which the substance/material will be analyzed and their comparison by taking measurements with a spectrophotometer. It allows analysis in the light of quantitative data rather than qualitative observation [11].

After the incubation, the tubes are checked with the naked eye, and the concentration of the substance that will prevent or reduce the growth is determined quantitatively by making measurements at a wavelength of 625 nm in the spectrophotometer compared to the control group. While it is expressed as the turbidity that can be easily distinguished by the eye when detected observationally, the concentration with a maximum reduction of 70% compared to the control as a result of the quantitative study is selected [11].

Table 3 A study of antimicrobial activity and the materials used [11]

	Agar Disc Diffusion	E Test	Serial Dilution	Time-Kill	Plaque Counting
Metals					
Ti6Al4V	+				+
Fe	+				+
Zn	+				+
Fe–Mn-Zn	+				+
Polymers					
PHB	+	+	+	+	+
PLA	+	+	+	+	+
PHB-PLA	+				
PMMA	+	+	+	+	+
PCL	+	+	+	+	+
PHB-V	+	+	+	+	
Ceramics					
N/M—HA	+				
TiO_2	+				
ZnO	+				
Composites					
Zn-TiO_2	+				+

2.4.3 Time-based death experiment (Time-Kill Method)

As a literal transfer of determination, it is preferred to determine the values with the log10 formation to have another and more straightforward representation from a scientific point of view. The fact that the Log10 formation has a value of 0.30 or less than the control group value proves that the polymeric substance has an inhibitory effect on at least half the number of microorganisms in that time zone. The number of cells is calculated after determining the absorbance values. After this process, with the help of a simple calculator, it is converted into a Log10 formation in terms of simple appearance and simple analysis [11].

2.4.4 Plaque counting method

The plaque counting method included in the study was performed only with metal-to-metal alloys, polymers, and composites according to the material condition (Table 3). The samples in the Petri dishes are incubated again at 37 °C for 24 h, and the antibacterial activities of the samples are evaluated by counting the number of colonies developed in the environment [11, 15, 17, 18].

3 Results

3.1 Agar disc diffusion method

The order and symbols of the strains used in the agar disk diffusion method are as follows (Figs. 1, 2, 3 and Table 4).

A)- (E.F.) *E. faecalis*, B) (S.T.) *S. typhi*, C) (S.E.) *S. epidermidis*,
D) (P.A.) *P. aeruginosa*, E) *E. coli,* F) (K.P.) *K. pneumoniae*, G) (B.S.) *B. subtilis*, H) (S.A.) *S. aeurous*, I) (C.A.) *C. albicans*

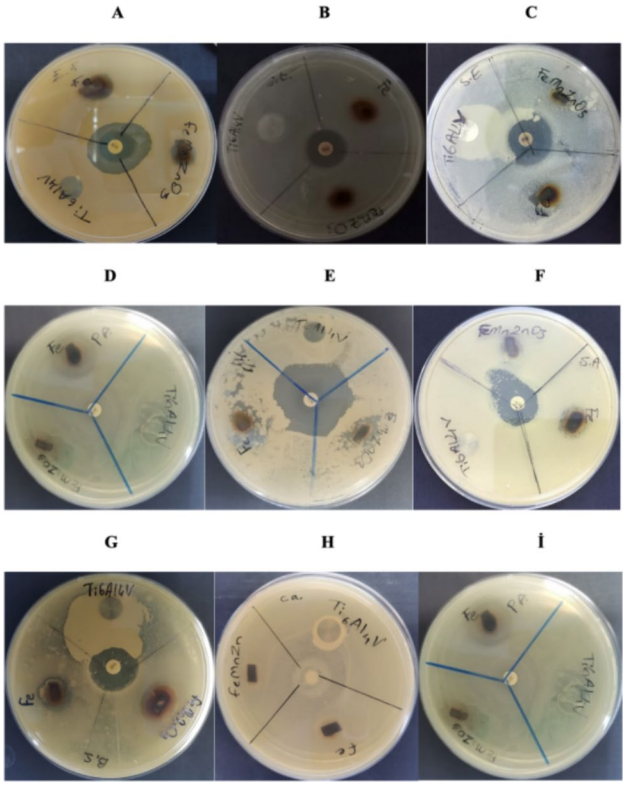


Fig. 1 Agar disc diffusion test of metal and metal alloy biomaterials, **A**) (E.F.) *E. faecalis*, **B**) (S.T.) *S. typhi,* **C**) (S.E.) *S. epidermidis,* **D**) (P.A.) *P. aeruginosa,* **E**) *E. coli,* **F**) (K.P.) *K. pneumoniae,* **G**) (B.S.) *B. subtilis*, **H**) (S.A.) *S. aeurous*, **I**) (C.A.) *C. albicans,* Ti6Al4V, Zn, FeMn (Iron manganese), FeMnZn (Iron Manganese oxide)

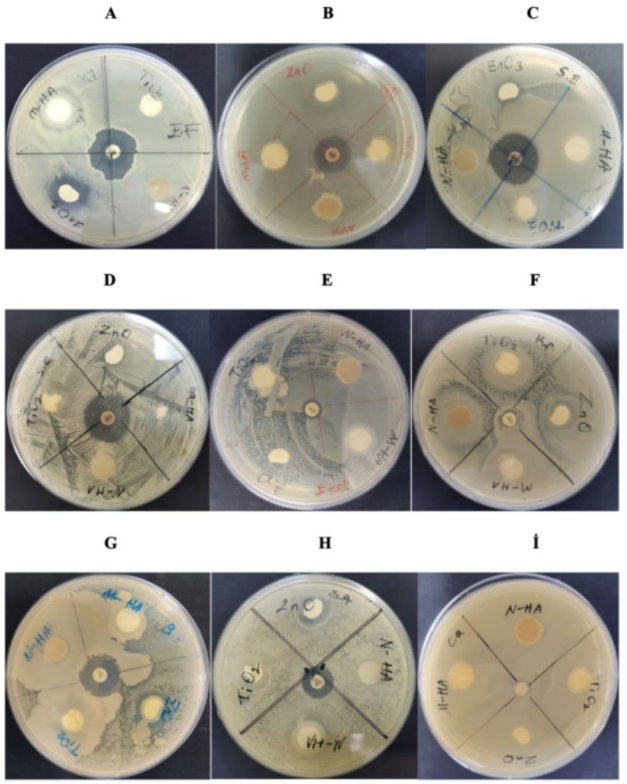


Fig. 2 Agar disc diffusion test of ceramic biomaterials, **A**) (E.F.) *E. faecalis*, **B**) (S.T.) *S. typhi,* **C**) (S.E.) *S. epidermidis,* **D**) (P.A.) *P. aeruginosa,* **E**) *E. coli,* **F**) (K.P.) *K. pneumoniae,* **G**) (B.S.) *B. subtilis*, **H**) (S.A.) *S. aeurous*, **I**) (C.A.) *C. albicans,* HA, TiO2, and ZnO (Hydroxyapatite, Titanium oxide and Zinc oxide)

All material groups were tested in agar disc diffusion experiments. Of these material groups, polymer and composite polymer biomaterials did not clearly show any antimicrobial activity effect. In addition, there was no evidence that an element arising from the characteristics and structure of the experiment affected the level of antimicrobial activity. The other material groups and the results of the antimicrobial activity of these groups and the effects for the formation, detection, or level of antimicrobial activity are as follows:

3.1.1 Metal and metal alloy biomaterials

The antimicrobial activities of metal and metal alloy-based biomaterials in our experimental studies were examined in the agar disc diffusion test method against all test microorganisms. As shown in Fig. 1.

According to the results of agar disc diffusion experiments of metal and metal alloyed biomaterials, the presence of an active substance that will show antimicrobial activity and prevent the reproduction of microorganisms has not been determined. Another remarkable result is that microorganisms have shown intensive reproduction around this biomaterial due to the structural form of Ti6Al4V metal and the material height, and it does not show any antimicrobial properties.

Regardless of the results in the images, when metal and metal alloy biomaterials are subjected to agar disk diffusion test method due to their own structures, oxidation occurs after contact with oxygen and liquid. Although they can show antimicrobial activity, it is also usual that the antimicrobial effect that may exist cannot be detected due to the chemical reaction and color formation caused by oxidation. Antimicrobial discs were placed in the image for the control sample in each experiment. The activities of these discs indicate that microorganisms react to any antimicrobial agent (Fig. 4).

3.1.2 Ceramic biomaterials

The antimicrobial activities of metal and metal alloy-based biomaterials in our experimental studies were examined in the agar disc diffusion test method against all test microorganisms. As observed in Fig. 2.

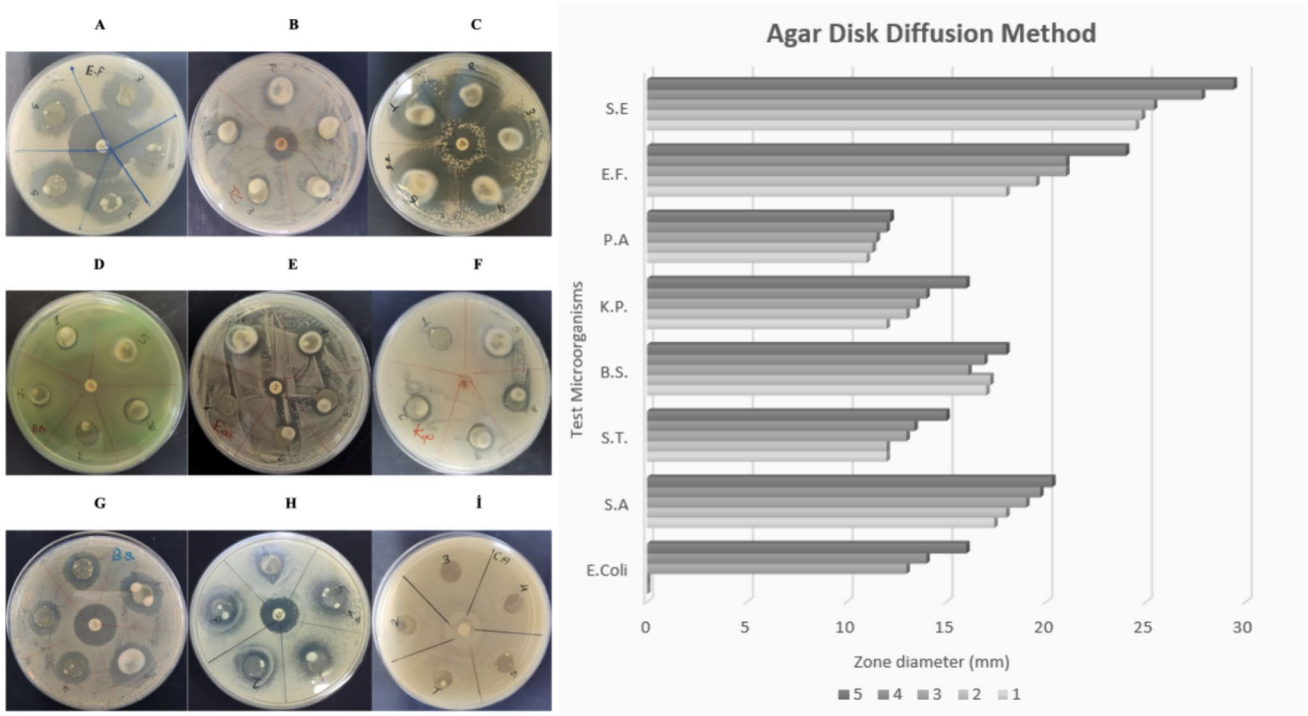


Fig. 3 Agar disc diffusion test of composite biomaterials and zinc metal, **A**) (E.F.) *E. faecalis*, **B**) (S.T.) *S. typhi,* **C**) (S.E.) *S. epidermidis,* **D**) (P.A.) *P. aeruginosa,* **E**) *E. coli,* F) (K.P.) *K. pneumoniae,* **G**) (B.S.) *B. subtilis*, **H**) (S.A.) *S. aeurous*, **I**) (C.A.) *C. albicans,* ZnTiO2 (ZincTitanium oxide), 1: Zinc, 2: Zinc + titanium, 3: increased zinc content by 3% of the material content, 4: increased zinc content by 6% of the material content, 5: increased zinc content by 9% of the material content

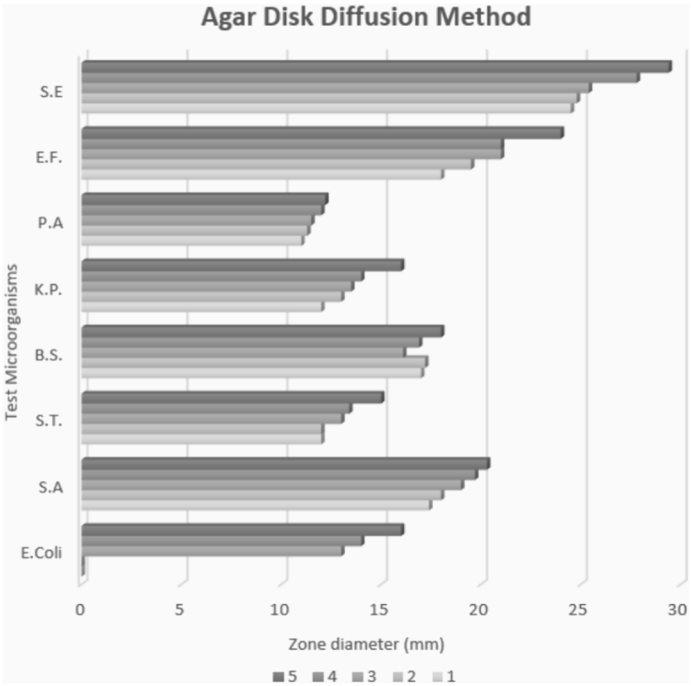
The situation differs slightly in ceramic biomaterials compared to the top two headings. This difference was first noticeable and gave a zone diameter of 14 mm against *E. faecalis*, 12 mm against *S. aureus,* and 10.5 mm against *S. epidermidis* among the nine microorganisms studied, respectively. As can be seen from the zone diameters, ZnO showed antimicrobial properties, but the remaining test microorganisms did not reveal any antimicrobial presence. No experiments have been detected in which ceramic biomaterials other than ZnO show antimicrobial activity. As an observational result of hydroxyapatite biomaterial, it has been shown that nano- or micro-sized does not affect based on antimicrobial activity property. Since ceramic biomaterials cannot be subjected to testing except for pressing into discs with a diameter of one cm, these efficacy data were analyzed in experiments by solidifying the powder ceramic biomaterials by external force by force. In line with this result, due to the lack of standardization of antimicrobial activity in biomaterials, it is unclear whether ZnO does not show antimicrobial activity to other microorganisms due to its introduction from powder form to solid form or repeated testing. As in every experiment, various antibiotic discs were placed in the middle of each petri dish to control microorganisms and antimicrobial agents. Although these discs have different zone diameters, they show that microorganisms can react to an antimicrobial agent.

3.1.3 Composite biomaterials

Biomaterials in the composite structure with metal matrix in our experimental studies and zinc metal have been tested with this group of materials because they have a zinc-based composite increase material. Antimicrobial activities were examined in the agar disc diffusion test method against all test microorganisms. As shown in Fig. 3. The materials in the image are **1**: Zinc, **2:** Zinc + titanium, **3:** increased zinc content by 3% of the material content, **4:** increased zinc content by 6% of the material content, **5:** increased zinc content by 9% of the material content.

Our study shows composite materials and zinc metal do not indicate an antimicrobial property against *C. albicans*. In addition, the first detail that catches the eye at the end of the experimental process is that the materials contributed in line with a specific correlation show antimicrobial activity. Only samples 1 and 2 were found to give any zone diameter in the E. coli strain. In addition, samples with the whole additive process were found to show high antimicrobial activity against all test microorganisms. When the photos of Petri dishes are taken, evaporation is observed because of the 24-h incubation period in the lower parts of the materials due to the metal composite type. Measurements were made considering the material conditions. As a result of the agar disc diffusion test method, both zinc metal and metal matrix

Table 4 Agar disk diffusion test zone diameters of composite biomaterials and zinc metal in Figure 3, ZnTiO2 (ZincTitanium oxide), 1: Zinc, 2: Zinc+titanium, 3: increased zinc content by 3% of the material content, 4: increased zinc content by 6% of the material content, 5: increased zinc content by 9% of the material content, (Standard deviation: ± SD)



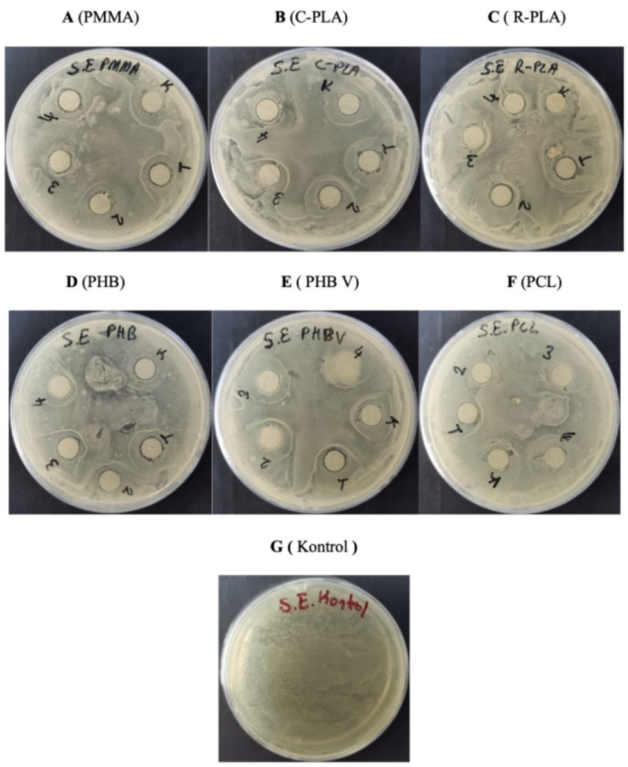


Fig. 4 E-Test test of polymers and copolymer biomaterials, S.E: S. epidermidis, 1: 50 mg/mL – 2: 25 mg/mL – 3: 12,5 mg/mL – 4: 6 mg/mL, PMMA (Poly(methyl methacrylate). C PLA (Resinex C Polylactic Acid), R PLA (Resinex C Polylactic Acid - For PLA was a made the same amount replay that's why it was coded as R(replay)), PHB (Polyhydroxybutyrate), PBHV (Poly(3-hydroxybutyrateco-3-hydroxy valerate), PCL (Polycaprolactone), Kontrol (Control)

composite materials were evaluated as a combination material group. In light of this evaluation, the test microorganisms that provide the highest anti-microbial rate are SE > EF > SA > BS > KP = EC > ST > PA.

Antimicrobial efficacy analysis in agar disc diffusion test method has been recorded as a method that obtains effective results in terms of both cheap cost and ease of application and low requirements. Within the scope of biomaterials, there was not much difficulty adapting polymer, metal, ceramic, and composite structured materials to the experiment under appropriate conditions and conditions. In this scope, a direct proportion of the findings regarding the suitability and feasibility of the materials to the investigation has developed. This correct proportion contributed to the development of appropriate correlation with the materials' use, structure, antimicrobial substance content, and susceptibility to the method. It has been shown that obtaining effective results by providing a specific stabilization of a wide variety of biomaterials under the same environment and experimental conditions can be included in a compelling analysis method within the scope of a particular standardization in biomaterial activity studies.

3.2 Analysis methods for determining the minimum ınhibition concentration

3.2.1 E-Test

Polymer and composite polymeric biomaterials In our experimental studies, it was investigated in the E-Test

method whether the polymer and composite polymer biomaterials were effective in terms of antimicrobial activity to the test microorganisms. The microorganism test results of the E-Test method, in which significant results were obtained, are shown 4. shown in figure.

When all the data were examined at the end of the entire experimental process, concentrations were used both above and below the concentration in the agar disc diffusion experiment of the biomaterials in which the active substance analysis was performed. In line with this result, E-test method was applied to all the test microorganisms. It has been observed that 50 mg/mL concentrations of PMMA, PHB, and PHB-V polymers against *S. epidermidis* microorganism give effect only to a small amount within the scope of E-test method. In the effective polymers' caliper and ruler measurement results, 12 mm zone diameter was measured for PMMA and 11 mm for PHB and PHB-V, respectively. No zone diameter and a measurement above 10 mm, i.e., disc size, which can be interpreted as antimicrobial properties of any concentration and bacteria other than *S. epidermidis* microorganism, was not observed.

Since the results of agar disc diffusion are known by not seeing the zone diameter and the results are obtained quickly, this test has shown that it will be an excellent part of the correlation when analyzed with other tests. The advantage of being a comprehensive Agar experiment, as well as its applicability and ease of analysis, play an effective role in both preferring the method and requesting effective results. If we change our perspective on the method regarding this result, it is very difficult to integrate biomaterial suitability into the test method. As a determining factor of this difficulty of the experiment, it is seen that only polymeric biomaterials from all biomaterial groups studied within the scope of this study are prone to this test and that the test is selective in the type of material to be analyzed rather than the ease of analysis. It is one of the most appropriate analysis methods for testing which types of biomaterials where dissolution is not a problem will determine from which concentration it exhibits antimicrobial activity. The most visible note from the end of this experimental process is that although the suitability of the biomaterial to be analyzed is limited, the E-test method plays a very active role in the realization process and the way of analysis, but it will also play a helpful role in terms of correlation between experiments.

3.2.2 Macro dilution to determine the minimum ınhibition concentration

Polymer and Composite Polymeric Biomaterials The antimicrobial activities of polymers and composite polymer biomaterials in our experimental studies were examined in the serial dilution test method against all test microorganisms. The experiment results are included after 24 h of incubation against the relevant test microorganisms of polymers and composite polymers.

In the light of the visuals and information in Tables 5 and 6, respectively, there are meaningful results in the serial dilution method, that is, the results that the substance/material has an inhibitory effect on the microorganism at a specific effective rate in line with the purpose of the test method.

The absorbance value obtained because of 24-h incubation of the C-PLA polymer at a 50 mg/mL concentration shows that the calculated cell count value inhibited more than half of the control sample. This value is considered the minimum concentration of inhibition.

In light of the absorbance values included in the tables, because of the calculations made using the table basis and formulation annexed to Table 7. The concentrations in 1 mL per hour, the number of cells, are calculated and included in the table. As a result of this calculation, because of the comparison with the control sample, the concentration amounts indicated in red in the tables show the MIC value for that microorganism. In short, at least half of the number of cells in the control sample is expressed as the concentration of the inhibitory substance, that is, MIC.$\mathbf{A} = \boldsymbol{\varepsilon c}\ell$

For example, it was determined by calculating the absorbance value and the number of cells in 1 mL that the most concentrated substance concentration of the C-PLA polymeric biomaterial, especially at the rate of 50 mg/mL, was both against other substance concentrations and that there was a visible turbidity difference in the 70–75% band, if

Table 5 Spectrophotometric results, *S. epidermidis,* Cell count CFU/mL X10^6, Incubation duration: 24h., Absorbance: 625 nm, PHB (Polyhydroxybutyrate), PLA (Resinex C Polylactic Acid) PCL (Polycaprolactone), PBHV (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), PMMA (Poly(methyl methacrylate)

Polymer / Number of Cells	PMMA	C-PLA	PCL	PHB	PHB-V	Concentration
Number of Cells	457	**170**	455	470	355	50 mg/mL
	421	435	422	443	481	25 mg/mL
	493	517	560	510	490	12.5 mg/mL
	507	535	520	541	546	6 mg/mL

(Standard deviation: ± SD)

Table 6 Spectrophotometric results, *S. aureus*, Cell count CFU/mL $X10^6$, Incubation time: 24 h., Absorbance: 625 nm, PHB (Polyhydroxybutyrate), PLA (Resinex C Polylactic Acid) PCL (Polycaprolactone), PBHV (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), PMMA (Poly(methyl methacrylate).

Polymer / Number of Cells	PMMA	C-PLA	PCL	PHB	PHB-V	Concentration
Number of Cells	406	**148**	466	374	297	50 mg/mL
	473	488	400	439	503	25 mg/mL
	525	530	547	528	472	12.5 mg/mL
	510	558	515	626	490	6 mg/mL

(Standard deviation: ± SD)

Table 7 Positive control exciton coefficient result for Time-Kill analysis method (Standard deviation: ± SD)

Duration/ Hours	Number of Cells ML / CFU 'C'	Absorption-625 OD	Extension Coefficient CFU X ML-1 X^{-1} 'E'
3	5.10^5	0,85	0.85 × 5.10–5
6	1.10^6	0,75	0.75 × 10–6
18	1.10^8	0,1	0.1 × 10–8
24	5.10^8	0,08	0.08 × 5.10–8

not in the 80–90% band, compared to the control sample. The serial dilution method has formed a basis for the time-kill method for detecting other polymeric biomaterials in the presence of an effect such as a short-term effect on the growth of microorganisms during the incubation process and then disappearance within 24 h. Concentrations of 50—25—12.5—6 mg/mL were used in this experimental process.

3.3 Time-based death test (time-kill method)

Polymeric and Composite Polymeric Biomaterials In Table 8, the number of cells calculated for spectrophotometric absorbance values at a wavelength of 625 nm in line with the test results of polymer biomaterials in concentrations ranging from 6–50 mg/mL against *S. epidermidis* test microorganism is given.

The fact that the Log10 formation has a value of 0.30 or less than the control group value is evidence that the

Table 8 Spectrophotometric results for Time-of-Death Analysis, *S. epidermidis,* Cell count CFU/mL $X10^3$ PHB (Polyhydroxybutyrate), PLA (Resinex C Polylactic Acid) PCL (Polycaprolactone), PBHV (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), PMMA (Poly(methyl methacrylate). (Standard deviation: ± SD)

	Polymer/Copolymers					
	PMMA	C-PLA	PCL	PHB	PHB-V	Concentration
	3. Hour					
Number of Cells	424	398	457	431	405	50 mg/mL
	417	437	492	398	435	25 mg/mL
	486	507	514	469	492	12.5 mg/mL
	479	466	509	501	478	6 mg/mL
	6. Hour					
	887	821	918	949	**323**	50 mg/mL
	931	899	958	837*	847	25 mg/mL
	959	926	1.084	980	893	12.5 mg/mL
	976	984	1.050	1.108	926	6 mg/mL
	18. Hour					
	87.800	93.400	75.100	99.000	81.000	50 mg/mL
	83.000	94.700	89.000	76.400	104.00	25 mg/mL
	105.500	131.500	88.300	92.000	100.700	12.5 mg/mL
	116.000	107.000	112.800	117.600	98.200	6 mg/mL
	24. Hour					
	457.500	**170.000**	455.000	470.000	355.000	50 mg/mL
	421.250	435.000	422.500	443.200	481.250	25 mg/mL
	493.750	517.500	560.000	510.000	490.000	12.5 mg/mL
	507.500	535.000	520.000	541.250	546.875	6 mg/mL

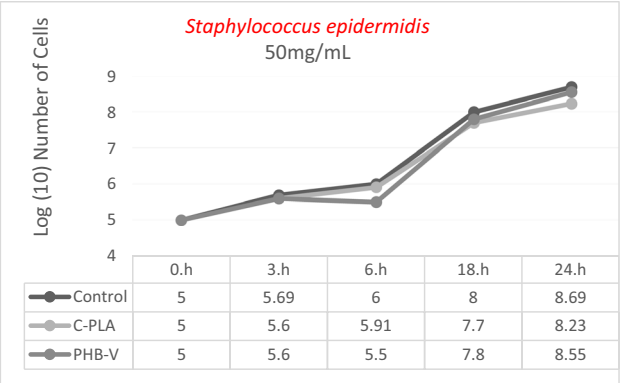


Fig. 5 Logarithmic value graph of 50 mg/mL concentration including C-PLA ((Resinex C Polylactic Acid), PHB-V (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), and Control group cell numbers against *S. epidermidis* bacteria

polymeric substance has an inhibitory effect on at least half the number of microorganisms belonging to that time zone—for example, 24. The clock control group has a value of 500,000,000. While this value is 8.69 in the log10 formation, half of this number of microorganisms, that is, 250,000,000, is 8.39 in the log10 formation. This state has the same condition for each concentration. The effect of inhibiting half of the bacteria against S. epidermidis was determined in 3 polymers at various times and concentrations compared to the control group. The cell counts in Table 8 are shown in log10 format shown in figure 5. As explained above, values with more than 0.30 difference in log10 form are indicated in black tone compared to the control group.

The time of death of C-PLA polymer against *S. epidermidis* bacteria at a concentration of 50 mg/mL was 24 h as shown in Fig. 5. The time of death of PHB-V copolymer against *S. epidermidis* bacteria at a concentration of 50 mg/mL was reported as 6 h in the same figure. Based on the positive control-based measurement control in Table 7 because of the time-kill analysis method performed simultaneously with other test samples against the *S. epidermidis* bacterial strain, it is stated in both charts and graphs that at least half of the cell numbers in the control group were inhibited at the time intervals mentioned above.

In Table 9, the number of cells calculated for spectrophotometric absorbance values at a wavelength of 625 nm following the test results of polymer biomaterials in concentrations ranging from 6–50 mg/mL against the *P. aeruginosa* test microorganism is given.

As observed in Fig. 6, the cell number values of polymeric biomaterials acting against the bacterium *P. aeruginosa* are shown in the Log10 formation. However, the following results have been obtained. As a result, the study detected the effect of inhibiting half of the bacteria against the bacterium *P. aeruginosa* compared to the control group in 2 polymers in various hours and concentrations. As stated in different aspects in other charts and graphs under the leadership of Table 9.

Table 9 Spectrophotometric results for Time-of-Death Analysis, *P. aeruginosa*, Cell count CFU/mL X10^3 PHB (Polyhydroxybutyrate), PLA (Resinex C Polylactic Acid) PCL (Polycaprolactone), PBHV (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), PMMA (Poly(methyl methacrylate). (Standard deviation: ± SD)

	Polymer/Copolymers					
	PMMA	C-PLA	PCL	PHB	PHB-V	Concentration
Number of Cells	3. Hour					
	432	480	470	487	476	50 mg/mL
	466	471	462	479	485	25 mg/mL
	459	502	487	512	526	12.5 mg/mL
	510	500	491	507	534	6 mg/mL
	6.Hour					
	807	898	966	948	880	50 mg/mL
	887	932	983*	982	920	25 mg/mL
	957	947	979	987	973	12.5 mg/mL
	961	964	1.022	1.198	995	6 mg/mL
	18.Hour					
	26.000	**29.000**	96.500	92.800	108,000	50 mg/mL
	103.300	81.500	98.60	126.60	116,000	25 mg/mL
	95.500	91.700	86.50	101.500	129,000	12.5 mg/mL
	116.900	124.400	133.400	147.300	118.200	6 mg/mL
	24.Hour					
	362.500	475.000	455.000	405.000	495.000	50 mg/mL
	470.000	515.500	470.000	510.000	522.500	25 mg/mL
	481.250	596.250	490.000	502.500	525.000	12.5 mg/mL
	550.000	585.000	572.500	585.000	620.000	6 mg/mL

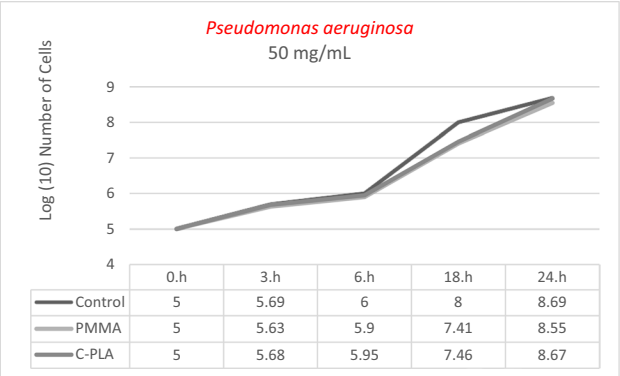


Fig. 6 Logarithmic value graph of 50mg/mL concentration of PMMA (Poly(methyl methacrylate), C-PLA (Resinex C Polylactic Acid), and Control group cell numbers against *P. aeruginosa* bacteria

It is stated in both charts and graphs that at least half of the time was inhibited at the times specified based on the positive control in Table 7 because of the time-kill analysis method performed simultaneously with other test samples against the *P. aeruginosa* bacterial strain. As a result of Fig. 6, the time of death of PMMA and C-PLA polymer against *P. aeruginosa* bacteria at a concentration of 50 mg/mL was determined at 18 h.

As result of experimental studies detected the effect of inhibiting half of the bacteria against *S. aureus* bacteria compared to the control group in 2 polymers at various clocks and concentrations. As stated in different aspects in other charts and graphs under the leadership of Table 10:

Following Fig. 7: The time of death of the C-PLA polymer against *S. aureus* bacteria at a 50 mg/mL concentration was determined within 24 h. The time of death of the PHB-V polymer against S. aureus bacteria at a concentration of 50 mg/mL was determined as of 18 h. Based on the positive control-based measurement control in Table 7 because of the time-kill analysis method performed simultaneously with other test samples against the *S. aureus* bacterial strain, it is stated in both the charts and graphs that at least half of the cell numbers in the control group were inhibited at the time intervals mentioned above.

Testing of polymeric biomaterials used because of the experiment at different concentrations revealed an effective analysis result in terms of antimicrobial properties. The most straightforward analysis advantage we have because of the Time-Kill test method is that it cannot or does not acquire in other ways, and the concentration difference is handled qualitatively and quantitatively. The Time-Kill test method is a method to determine the accuracy or inaccuracy of methods in which antimicrobial properties in biomaterials are analyzed observationally. Suppose there is only an observational analysis, as in agar disk diffusion methods. In that case, the time-kill test method determines the time interval and the number of live and dead microorganisms in which this effect occurs.

Table 10 Spectrophotometric results for Time-of-Death Analysis of *S. aureus*, Cell count CFU/mL X10^3, PHB (Polyhydroxybutyrate), PLA (Resinex C Polylactic Acid) PCL (Polycaprolactone), PBHV (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), PMMA (Poly(methyl methacrylate). (Standard deviation: ± SD)

	Polymer/Copolymers					
	PMMA	C-PLA	PCL	PHB	PHB-V	Concentration
	3. Hour					
Number of Cells	390	356	461	411	348	50 mg/mL
	410	319	508	379	365	25 mg/mL
	445	442	503	452	484	12.5 mg/mL
	470	483	539	440	433	6 mg/mL
	6.Hour					
	820	608	808	748	593	50 mg/mL
	855	839	860	822	757	25 mg/mL
	917	791	812	991	763	12.5 mg/mL
	968	834	954	1.034	946	6 mg/mL
	18. Hour					
	77.800	63.400	72.800	59.700	**31.800**	50 mg/mL
	81.000	94.700	122.100	88.500	92.400	25 mg/mL
	95.000	99.210	98.550	82.600	105.100	12.5 mg/mL
	106.000	117.460	101.300	97.100	87.690	6 mg/mL
	24. Hour					
	406.000	**148.750**	466.000	374.600	297.500	50 mg/mL
	473.000	488.125	400.000	439.100	503.800	25 mg/mL
	525.000	530.625	547.500	528.700	472.000	12.5 mg/mL
	510.000	558.125	515.650	626.500	490.200	6 mg/mL

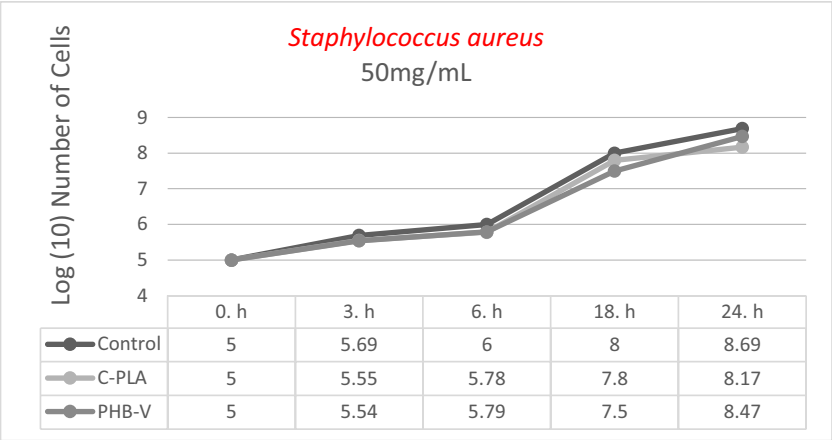


Fig. 7 Logarithmic value graph of 50 mg/mL concentration of C-PLA ((Resinex C Polylactic Acid), PHB-V (Poly(3-hydroxybutyrate-co-3-hydroxy valerate), and Control group cell numbers against *S. aureus* bacteria

On the other hand, if the biomaterial has antimicrobial activity by observational analysis methods, the time-kill test method is a method that prevents misinterpretation since the existing effect cannot show itself at the end of 24 h (after an average overnight incubation). Suppose the biomaterial is used in any composite structure. In that case, it will play an active role by transferring its antimicrobial effect but cannot show at the end of 24 h to the composite biomaterial. This method is essential to prevent possible errors in interpreting the results. In short, it is a detailed analysis tool to determine to what extent our conclusion about the antimicrobial activity of biomaterials is correct and how much it can be improved.

As a result of the values, there was no net decrease in the number of bacteria (inhibiting half) in the number of bacteria in copolymers and polymers against *E. faecalis, S. typhi, E.Coli, P. aeruginosa, S. epidermidis B. subtilis* and *C. albicans* microorganisms at any time and concentration value compared to the control group.

3.4 Plaque colony counting method

3.4.1 Polymer and composite polymer biomaterials

Since no clear antimicrobial separation is observed as a result of plaque counting of polymeric biomaterials if it is to be expressed orally instead of the table, the PCL polymer against microorganisms has been observed to grow fewer colonies in the petri dish in the petri dish in numbers than the control samples (This observation is that the colonies are less and the statements due to the inability to perform the counting stage are made quantitatively). It was also found that the PCL polymer produced fewer colonies in the petri dish and the PHB polymer in the petri dish compared to the control samples. Apart from these polymers, which showed some undergrowth against control samples, no finding could be interpreted as any antimicrobial effect in the remaining polymers and microorganism tests. As a result of this analysis, since it was not concluded that the antimicrobial effects of the polymer did not exist at all, as stated in other methods, it was once again revealed that changes in the amount and density of the content could affect this property representation (Table 11).

3.4.2 Metal and metal alloy biomaterials

The first striking point in the results of the plaque counting method of metal and metal alloy biomaterials was that the newly produced FeMnZnO biomaterial made a noticeable difference in terms of antimicrobial properties against both Ti6Al4V and Fe material, especially the control sample against all microorganisms. In addition, another effective result is that both FeMnZnO and pure Fe show a clear antimicrobial property and do not allow any microorganism (*E. coli)* colony to grow. Thanks to these effective findings, it

Table 11 Plaque Counting method, Colony numbers of polymeric materials, PCL (Polycaprolactone), PHB (Polyhydroxybutyrate), PMMA (Poly(methyl methacrylate), PLA (Resinex C Polylactic Acid), (Standard deviation: ± SD)

E. faecalis	PCL	215
	PHB	> 468
	PMMA, C-PLA, Control	> 750
S. typhi	PCL	> 481
	PMMA, C-PLA, PHB, Control	> 750
S. epidermidis	PCL	> 520
	PHB	162
	PMMA, C-PLA, Control	> 750

(Standard deviation: ± SD)

Table 12 Plaque Counting method, Colony numbers of Metal and Metal alloy materials, Fe-Mn (Iron-Manganese), Ti6Al4V (Titanyum Grade 5 (%90 titanyum, %6 alüminyum ve %4 vanadyum)), Fe (Iron), (Standard deviation: ± SD)

E. faecalis	Fe–Mn	243
	Control – Ti6Al4V- Fe	> 750
S. typhi	Fe–Mn	> 277
	Control – Ti6Al4V- Fe	> 750
E. coli	Fe—Fe–Mn	0
	Control – Ti6Al4V	> 750

(Standard deviation: ± SD)

was determined that FeMnZnO biomaterial has a superior antimicrobial activity against these microorganisms, especially in the form of *E. faecalis* > *E. coli* > *S. aureus*. While pure iron has a superior antimicrobial activity against E. coli according to the biomaterial content (Table 12).

3.4.3 Composite biomaterials

The materials listed in the table are **4**: Zinc metal, **8**: Zinc + Titacum oxide, **12**: increased zinc content by 3% of material content, **16**: increased zinc content by 6% and **20:** increased zinc content by 9%.

As a result of the plaque counting method, the antimicrobial activity rates of composite materials and zinc metal, considering the experimental purpose, that is, the number of colonies that can grow in petri, are given in Table 13. The colony numbers in the table are determined as C*FU*/mL.

As a result of composite biomaterials and zinc metal plaque counting experiments, the first striking point is that with the increase in the additive rates in the material groups (12, 16, 20), no bacterial colonies were detected in the Petri dishes of all the test microorganisms. A remarkable decrease in the number of bacterial colonies was detected in all the strains studied respectively (4, 8). These results showed evident antimicrobial activity in all material groups with a content ratio of 12 and greater than 12. In the experimental images, it was determined in the detailed microscopic analysis that all test microorganisms formed some dense breeding areas, especially in sample number 4, and that these regions were inoculum and microorganism-derived colony groups that were briefly overlapped without any contamination. A linear correlation was observed in the antimicrobial activity rates of various doped materials of this metal matrix composite material group following an increase in the additive rate. This linear correlation is of great importance in clarifying the time stabilization of the antimicrobial activity display of the studied material group in the process, advancing the study within the scope of various microorganisms and studying new material groups.

Table 13 The number of colonies of composite materials and zinc metal in petri dishes because of the plaque counting method, (E.F.) *E. faecalis*, (S.T.) *S. typhi,* (S.E.) *S. epidermidis,* (P.A.) *P. aeruginosa, E. coli,* (K.P.) *K. pneumoniae,* (B.S.) *B. subtilis*, (S.A.) *S. aeurous*, (C.A.) *C. albicans, 4: Zinc, 8: Zinc + titanium, 12: increased zinc content by 3% of the material content, 16: increased zinc content by 6% of the material content, 20: increased zinc content by 9% of the material content*

Sample No / Strains	4	8	12	16	20
EF	> 310	0	0	0	0
ST	88	7	0	0	0
SE	104	1	0	0	0
PA	> 589	11	0	0	0
EC	> 117	2	0	0	0
KP	35	18	0	0	0
BS	56	3	0	0	0
SA	2	0	0	0	0

(Standard deviation: ± SD)

4 Discussion

4.1 Antimicrobial activity method based analysis

Within the scope of this study, antimicrobial activity analysis methods were evaluated on the positive aspects of the methods themselves in terms of analysis and the negative aspects they are affected by, the examination of linear or nonlinear correlations between them and other methods, and the research results of biomaterials subjected to antimicrobial activity methods.

4.2 Agar disc diffusion method

The agar disk diffusion method is the least affected by the structural form and physical state of the tested material. For example, the biomaterials in our study include materials in powder form or solid form; if the materials in powder form have solubility, they are impregnated into sterile disks after dissolution and tested. If it is not soluble, the powder sample is pressed into solid disks. The agar disk diffusion method has been determined as the method in which elements such as surface properties and thickness of this solid are tested under the most flexible conditions.

In addition, since the ceramic materials are in powder form and their solubility is not clear, only the agar disc diffusion method was suitable for analysis among the methods made within the scope of this study. As a result of the investigation, there are negative aspects of working with different types of materials that are the positive side of the method when the evaluation is made. For example: Negative effect is that oxidation can be observed because of contact

with oxygen and liquid depending on the metal basis of the materials used. This is not a favorable situation for effective zone diameter measurements. Starting the experiment when the surfaces of such materials are very clean and in the desired condition and by keeping the test time to a minimum (18–24 h) and obtaining the results before from the deformation of the material can lead the researchers to the healthiest results.

The agar disc diffusion method showed a linear correlation with the plaque counting method regarding its results according to the material type. For example: When the effects of composite materials and metal materials from which clear measurements cannot be obtained due to material type are followed up with the plaque counting method, it is observed with clear data that agar disc diffusion constitutes a leading working group for plaque counting studies despite the material-induced negativity in the method. The simultaneous operation of these methods when the material type allows gives a high level of clarity and robustness of the results. As a result, although the Agar disc diffusion method is traditional, well-known, cost-inexpensive and wide range of materials, the method and the in-material disadvantages do not fully foresee that the method can give a clear efficiency according to the material type.

4.3 Minimum inhibition concentration determination methods

The fact that the determination of the minimum inhibition concentration has different two-based methods provides a source of deep analysis and the possibility of effective mutual interpretation of the results.

4.3.1 E test method

In studies such as Berghaus et al. [19] or Gupta et al. [20], the e-test method is based on observational results by impregnating a test strip with an active substance. However, in our study, Unlike the known e test studies in the E test method, it is based on the observational results made by impregnating an active substance in a single test strip, but based on which disc the zone diameter is also seen because of impregnating different concentrations of substances (50–25-12.5–6) into the filter discs. This is the only difference from the classical method.

The advantage of this situation is since researchers, who have low laboratory and material resources, can reach the desired goal, the presence or absence of antimicrobial activity, in the easiest way. Polymer and copolymer materials within the scope of the study are included in this method due to the advantage of solubility. Since the E test method is a group of methods where materials with active solubility status will get results, there is no close range of materials with methods other than time kill and serial dilution method. As a result, it was determined that the method actively gave results in the case of different concentrations of the material in the process that were described as MIC. When the material type of these methods is also possible, working simultaneously gives a superior rate in terms of clarity of results.

4.3.2 Spectrophotometric analysis after serial dilution

The serial dilution method, another MIC determination method, was found to be in correlation with the E test. The fact that this correlation is the method that consolidates the results of the experiment and quantitatively takes the analysis to the next level is one of the most obvious advantages of the method. Within the scope of our study, it was determined that the serial dilution method should be evaluated as an analysis method after 24 h of incubation by taking absorbance values using spectrophotometers and determining the number of cells belonging to that moment in the tube and the sensitivity of this test method and the preliminary study of e test in such material groups. For example, testing the result obtained in the E-test method in the serial dilution method and not being included in the E- test method or the failure to determine the effectiveness of the substance based on that method is an apparent supporting factor to prevent erroneous decision-making situations.

The fact that the MIC calculation is examined because of 24th-hour incubation based on the method is an open issue that substances that are not very effective and continuous in this process cannot prevent the reproduction of microorganisms as a result of the 24th Hour, but this can occur in different parts of the incubation process. Considering this situation, just as the E-test method supports the serial dilution method as a pioneering study, the serial dilution method sheds light on the issue of which microorganism and substance pioneered the time-kill method and which microorganism and substance should be investigated at a higher depth.

4.4 Time-based death test (time-kill method)

Results were analyzed based on colony counting in Petri dishes with agar. The difference in our study is that the measurements were made at 625 nm with spectrophotometric analyzes at every hour interval. Examining the substances dissolved in polymer or any solvent with this method saves time and reduces the workload.

This method is highly preferred in the analysis of substances tested in various bacteria and candida species [21]. Suppose the importance of this method is to be emphasized. In that case, the spectrophotometric measurements in the findings section also show us that the tested materials show possible antimicrobial properties within certain time zones

and cannot be transferred to future time zones. Analyzing this separation by spectrophotometric measurement lets us know which biomaterials can show a more effective antimicrobial activity when used in various composite structures. For example, the antimicrobial activities of polymer biomaterials such as PLA, PHB, PMMA, which show positive results when used in multiple composite structures at different rates and amounts, may contribute to the newly formed biomaterial [11].

As stated in the above heading of the time-dependent death test, E test, serial dilation, and time-kill studies are analysis methods that are calculated at different depths in the exact parallel. One of the superior advantages of the time-kill method is that two different quantitative pathways can lead to the result within the technique. In addition to using absorbance values as in the scope of this study, mathematical data is obtained by counting the colonies because of incubation by sowing agar petri after each period. This advantage is that the method is suitable for the laboratory facility where it will be studied while dealing with the solubility status as well as E-test and serial dilution in terms of material, which allows it to be positively differentiated from other methods. In short, the result can be achieved clearly by differentiating the outlet without changing the method.

As a result of experiments and observations, it has been determined that the MIC concentration or other concentrations after MIC can be effective alone in examining the efficacy possibilities, in which time it provides simultaneous results with the methods that provide linear correlation.

4.5 Plaque counting method

The plaque counting method is unsuitable for other analysis methods rather than the solubility state, for example, metal-based materials that cannot be measured based on material type in the agar disc diffusion method or a solid material or a substance that cannot dissolve in general. However, it can dissolve and has a clear separation by efficiently and effectively descending the analysis of the material with a smooth surface based on the number of cells.

The analysis presents a linear correlation with the agar disc diffusion method and a co-based study based on a clear antimicrobial activity analysis. In cases where the agar disc diffusion method is insufficient in sensitivity, plaque counting is an effective method to fill this deficiency. It is a clear analysis method that allows the analysis of the effectiveness of polymeric biomaterial, which does not give any zone diameter in metal-based materials or agar disc diffusion method, but whose differences we observe in terms of the number of colonies. The plaque counting method is based on the principle that the materials that give the most information to the researcher compared to other agar methods clearly show both the effect change between microorganisms and the effect change of the materials in a microorganism on a colony-based basis. As in every experimental study, in the plaque counting method, the most preferred materials, which can show antimicrobial properties, are tried to be selected and tested by the researchers [11, 18, 22, 23].

Sun et al. [18], carried out the antimicrobial activity of the material with the plaque counting method in their research. Another study in which the same procedure was preferred in the first place was conducted by Sotoudehbagha et al. [15] In these studies, the extent of the antimicrobial activity of the material was investigated in detail. However, this study aimed to show how a biomaterial should be followed by keeping the easiest method and research possibilities at the most basic level. Because one of the first properties that the investigated materials should provide in terms of being biomaterial is that they have the necessary antimicrobial activity, in this study, it has been demonstrated that the presence or absence of antimicrobial activity can be achieved by the plaque counting method in the most fundamental principle and the most accessible situations.

5 Conclusion

The diversity of areas where biomaterials are used in line with the need has reached very high levels today. Antimicrobial properties are an area where the researcher can be mistaken and misinterpret the properties of the biomaterial. Our study tested the antimicrobial activity of different types and structural properties of biomaterials. These test methods were selected from the most common and uncomplicated methods.

In addition, various strains of bacteria and fungi were used to investigate the response of these antimicrobial activity tests to different microorganisms. The vision and motivation behind our work is to create a set of analyses that innovate, improve and solve problems.

One of the results obtained within the scope of this purpose is the determination that there is no standard for the solution and inoculation amounts used during the testing of biomaterials, although the same methods are used in the sources cited from the literature in the discussion section (agar disk diffusion and plate counting method section), and an innovation was developed against this. Rather than biomaterials researchers seeing these and other differences when they look at the literature, we wanted to prepare an understandable and clear analysis scheme. One of the basic principles and one of this study's most practical features is establishing a standard that will eliminate this uncertainty and diversity when testing biomaterials. This aim is envisaged to provide a new perspective for biomaterials, which have recently increased in use, to contribute to the literature and support researchers.

From a different point of view in our study, analyzing different results of some soluble biomaterials (polymers and copolymers) in different tests helps explicitly us to reveal an innovation for biomaterial studies. To avoid situations where researchers are unable to detect the antimicrobial activity of biomaterials, even in small amounts, when appropriate tests are not selected, we wanted to bring a different perspective to the studies of researchers. For this purpose, the correlations of antimicrobial activity tests and the levels of complementing each other's deficiencies were investigated, analyzed, and evaluated. This result shows us that there is an opportunity to eliminate the situation of not being able to detect existing antimicrobial activity and not using a potential antimicrobial additive unless a specific antimicrobial test method is selected.

For instance, the newly synthesized $ZnTiO_2$ composite biomaterial shows effective antimicrobial activity against almost all test microorganisms. It shows how such created biomaterials will be subjected to antimicrobial activity testing. This study, in line with the detailed antimicrobial activity test results, enabled the determination of the usage areas of such materials and led to more detailed studies. Among the other active group polymer materials, PLA polymer showed antimicrobial activity depending on how detailed the method was (Time-kill method). Other polymers could not carry their activity to the end of the 24th Hour. When all these polymers are increased in quantity, they can produce more effective results when used in various composite structures and when it is the subject of detailed research.

The main challenge for researchers conducting biomaterial or antimicrobial activity experiments is the start-up phase. The initial stage is finding the most accurate one from the many options that can be chosen. Let's look at the studies on the in vitro antimicrobial activity of biomaterials in this study from a different point of view. It is to help any biomaterials researcher carry out experimental studies in the easiest way. This research creates a basis for researchers to select microorganisms and tests easily.

The antimicrobial activity tests of all biomaterial groups included in the study are considered as support and suggestions for researchers to avoid choosing the wrong method and study area. Choosing the wrong method or not knowing which methods show a linear correlation with each other if more than one method is to be studied allows a possible antimicrobial biomaterial to be overlooked. This study was carried out to help researchers in the more difficult part than designing the biomaterial. Because in addition to the structural state of biomaterials it is necessary to know the strengths and weaknesses of the antimicrobial activity methods to be used. This information shows how methods can be linked to each other.

Antimicrobial activity studies are a field with basic analysis techniques that appeal to multidisciplinary studies. In order to convey every detail to a researcher who will work in this field and to get full performance from biomaterials and methods, the path to be followed should be mapped. Our study can be considered as forming all the motifs and framework of this map. The most different and most necessary motivation tool in our study is that it should be known that the compatibility between the antimicrobial activity studies of biomaterials is as perfect as the key-lock match and it is single solution-oriented.

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Declarations

Conflict of Interest The authors have no competing interests to declare that are relevant to the content of this article.

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