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Determination of the antimicrobial and antibiofilm activity of lyophilized cornelian cherry (*Cornus mas* L.)

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ABSTRACT: This study investigates the antimicrobial and antibiofilm activities of lyophilized Cornelian cherry (*Cornus mas* L.) and its methanol extract against a range of clinically significant pathogens. The research aimed to evaluate the potential of these natural compounds as alternative antimicrobial agents, given the global rise in antimicrobial resistance. Antibacterial activity was assessed using agar well diffusion and broth microdilution methods, while antibiofilm efficacy was determined through biofilm inhibition assays. The methanol extract exhibited broad-spectrum antibacterial activity, effectively inhibiting all tested bacterial strains, whereas the lyophilized form demonstrated no activity against *Klebsiella pneumoniae* and *Escherichia coli*. Both forms showed strong biofilm inhibition, particularly against *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, with inhibition increasing in a dose-dependent manner and peaking at the minimum inhibitory concentration. No antifungal activity was observed against the tested *Candida* species. The superior efficacy of the methanol extract may be attributed to a higher concentration of phenolic and anthocyanin compounds. These findings suggest that Cornelian cherry, particularly in its methanol-extracted form, holds promise as a natural antimicrobial and antibiofilm agent. Further research is warranted to explore its potential applications in clinical and industrial settings.

KEYWORDS: Cornelian cherry; *Cormus mas* L.; antimicrobial effect; antibiofilm effect

1. INTRODUCTION

Cornelian cherry (*Cornus mas* L., CM) is a small tree or shrub, 3 - 9 meters high, belonging to the Cornaceae family. It is known that there are approximately 50 cornelian cherry plant species in the world [1]. Since the time of Virgil and Pliny (70-19 BC), CM has been growing in Southern Europe and Southwest Asia, spreading from the Caucasus to Türkiye, Romania, Bulgaria, Italy and the central Europe [2, 3]. The fruits of the CM plant, which tastes sour, are olive-sized and vary in color as purple, yellow or red [4]. It bears fruit in September - October and the length of the fruits is 10 - 23 mm and the weight is 1 - 10 g, while the seed weight is between 0.19 - 0.59 g [5]. CM is rich in ascorbic acid, anthocyanins, phenolic compounds, flavonoids, malic acid, tartaric acid, citric acid, gallic acid, chlorogenic acid and loganin [6]. While various anthocyanins and numerous phytochemicals are present in different segments of CM, the primary anthocyanin groups identified in CM consist of cyanidin 3-O-galactoside and pelargonidin 3-O-galactoside [3, 7, 8]. CM contains significantly more vitamin C than fruits such as strawberries and lemons. Vitamin C content varies between 34 - 100 mg/100 g fresh weight [1]. Modern pharmacological studies have shown that CM has several biological properties such as antidiabetic, lipid-lowering, antioxidant, anti-inflammatory, antibacterial, anticancer, anticoagulant, antiparasitic effects and protective effects on liver and kidney function [9-11]. It was found that CM was protective against *S. cerevisiae* and that CM extract showed antimicrobial activity against *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* strains [12, 13]. The properties of extracts and ferments obtained from CM and their potential use in the treatment of dermatological conditions were

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compared with cosmetic products and it was observed that CM showed antibacterial activity depending on the concentration and fermented CM showed greater antibacterial activity [14].

Antimicrobial resistance is a rapidly increasing global issue that can make treating infectious diseases difficult or even impossible [15]. It is important to address this problem to ensure effective treatment options remain available. Biofilm is an extracellular matrix layer formed by microorganisms adhering to living or non-living surfaces. This layer consists of polysaccharides, DNA, microorganisms, and proteins. Microorganisms in biofilm are not affected by antimicrobial drugs and can develop resistance to them. Infections with biofilms in the body are difficult to treat and can become chronic, potentially spreading throughout the body. The discovery of new antimicrobial and antibiofilm compounds is crucial for human health. Plants, with their rich chemical content, have significant potential for producing effective molecules that can prevent or reduce biofilm development and fight infectious diseases [16-20].

Studies investigating the health effects of CM and the effect of lyophilized (Lyp) methods on nutritional value and phenolic compounds can be found in the literature [5, 11, 21]. However, no studies investigating the antibacterial effect of Lyp CM on a large scale were encountered. In our study, antimicrobial and antibiofilm activities of *Cornus mas* L. samples were investigated against microorganisms, which are frequently encountered infectious agents in humans.

2. RESULTS

2.1 Antimicrobial effect

In agar well diffusion test results, methanol extract (MetE) showed antimicrobial activity against all tested bacteria, whereas Lyp CM showed antimicrobial activity against all bacteria except *E. coli* and *K. pneuminoae*. Both samples did not show antimicrobial activity against the yeasts tested (Table 1). When the two samples were compared, it was observed that the MetE had a higher level of antibacterial activity (Figure 1).

2.2 Antibiofilm effect

The Lyp form of CM and its MetE exhibited strong biofilm inhibition rates, particularly against *S. aureus*, *E. faecalis*, *S. pyogenes*, *P. aeruginosa*, and *A. baumannii* strains. This inhibitory effect was dose-dependent, reaching its highest level at the minimum inhibitory concentration (MIC). In contrast, the biofilm inhibition values for other microorganisms were found to be lower (Table 2).

2.3 Anthocyanin content

Total anthocyanin was found as 1187.77 ± 4.53 mg per 100 g.

3. DISCUSSION

Antimicrobial resistance (AMR) poses an escalating global threat, with projections from the World Health Organization (WHO) estimating that by 2050, AMR could lead to 10 million deaths annually [22]. This challenge is exacerbated by the ability of microorganisms to form biofilms, which confer enhanced resistance to antimicrobial agents and complicate infection management [23, 24]. Biofilms, complex microbial communities embedded within self-produced extracellular polymeric substances, are implicated in persistent infections and are notoriously difficult to eradicate [25]. Addressing this issue necessitates the exploration of novel antimicrobial and antibiofilm agents, with plant-derived compounds offering promising avenues due to their diverse bioactive profiles [26].

Our study sought to investigate the antimicrobial and antibiofilm activities of Lyp CM and its MetE against a spectrum of clinically relevant pathogens. The findings revealed that both Lyp CM and its MetE exhibited notable antibacterial activity, albeit with varying efficacy across different microbial strains. Notably, the MetE demonstrated a broader antimicrobial spectrum, inhibiting all tested bacterial strains, while the lyophilized form was ineffective against *K. pneumoniae* and *E. coli*. Neither preparation exhibited antifungal activity against the tested *Candida* species. Similar findings have been reported by Çömlekçioğlu et al. (2022), Yiğit (2018), Krzyściak et al. (2011), and Krisch (2008), emphasizing the antimicrobial potential of CM extracts [27-30].

Table 1. Agar well diffusion and microdilution test results of Lyp and MetE CM

	Agar well diffusion test				Microdilution test	
	Inhibition zone diameter (mm)				MIC (mg/ml)/ MBC (mg/ml)	
	Lyp	MetE	Mrp	AmB	Lyp	MetE
Pseudomonas aeruginosa ATCC 27853	6.93±0.04	14.17±0.05	33.53±0.33	-	1.56/12.5	0.98/7.81
Pseudomonas aeruginosa PAO1	6.4±0.05	14.27±0.21	29.48±0.27	-	1.56/12.5	0.78/3.91
Staphylococcus aureus ATCC 43300	9.24±0.03	14.6±0.06	31.02±0.18	-	1.56/12.5	1.96/7.81
Klebsiella pneumoniae ATCC 4352	0	4.67±0.07	33.43±0.32	-	-	15.63/62.5
Proteus mirabilis ATCC 14153	10.09±0.15	15.92±0.04	34.32±0.25	-	12.5/100	15.63/62.5
Salmonella typhimurium ATCC 14028	7.63±0.06	16.32±0.17	34.92±0.14	-	3.13/25	1.96/7.81
Staphylococcus epidermidis ATCC 12228	11.48±0.03	15.35±0.03	51.52±0.22	-	1.56/3.13	1.96/3.91
Acinetobacter baumannii ATCC 19606	8.63±0.08	14.16±0.10	33.13±0.27	-	1.56/12.5	3.91/31.25
Enterococcus faecalis ATCC 29212	3.78±0.03	14.55±0.06	19.94±0.11	-	6.25/50	3.91/15.63
Staphylococcus aureus ATCC 29213	7.29±0.06	14.45±0.07	37.05±0.17	-	12.5/50	15.63/62.5
Staphylococcus aureus ATCC 25923	8.61±0.03	13.81±0.03	36.84±0.21	-	1.56/12.5	0.49/0.98
Streptococcus mutans ATCC 25175	3.58±0.06	9.05±0.09	13.43±0.19	-	12.5/100	31.25/250
Streptococcus pyogenes ATCC 19615	7.30±0.07	18.07±0.05	58.47±0.35	-	0.39/1.56	0.98/3.91
Escherichia coli ATCC 25922	0	10.57±0.07	35.11±0.34	-	-	1.96/15.63
Candida albicans ATCC 90028	0	0	-	24.77±0.19	-	-
Candida tropicalis kuen 1021	0	0	-	19.79±0.22	-	-
Candida glabrata ATCC 90030	0	0	-	25.36±0.34	-	-
Candida albicans ATCC 10231	0	0	-	27.28±0.23	-	-

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, Lyp: Lyophilized, MetE: Methanol extract, Mrp: Meropenem, AmB: Amphotericin B,: Not done

The enhanced antimicrobial efficacy of the MetE may be attributed to the increased solubility and extraction efficiency of phenolic and anthocyanin compounds, which are known to exert potent antimicrobial effects [26]. This observation aligns with previous reports highlighting the superior antimicrobial activity of MetE phytochemicals [31, 32]. The variation in susceptibility between Gram-positive and Gram-negative bacteria observed in this study is consistent with the structural differences in their cell walls. Gram-negative bacteria possess an outer membrane that can impede the penetration of antimicrobial agents, potentially explaining the diminished efficacy of the Lyp form against *E. coli* and *K. pneumoniae* [30].

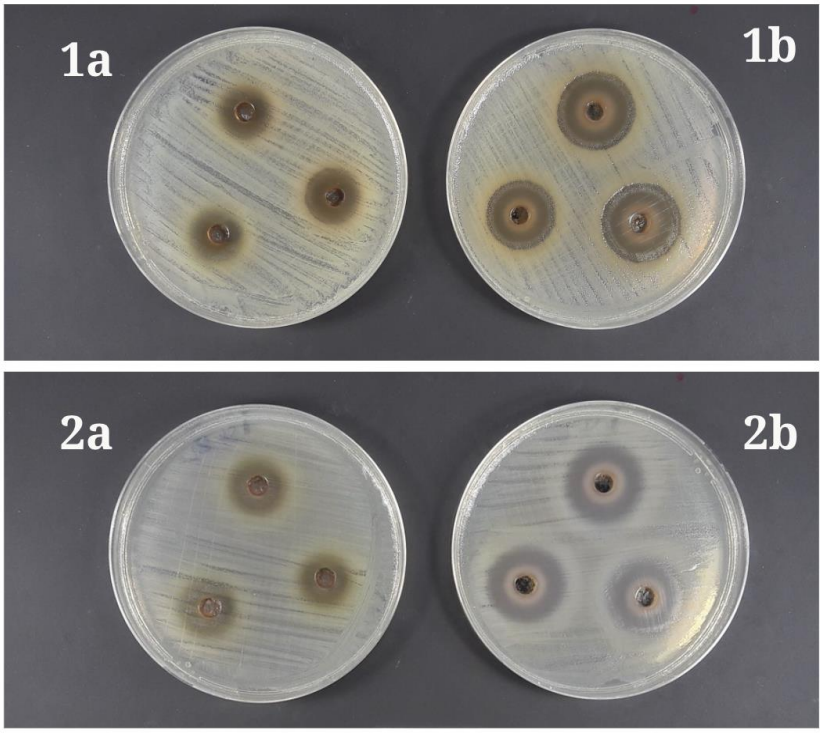

Figure 1. Agar well diffusion. 1a – Antimicrobial effect of lyophilized CM (1a) and methanol extracted CM (1b) on *S. aureus* ATCC 43300; antimicrobial effect of lyophilized CM (2a) and methanol extracted CM (2b) on *S. typhimurium* ATCC 14028.

In terms of antibiofilm activity, both forms of CM demonstrated significant inhibitory effects, particularly against *S. aureus*, *E. faecalis*, *S. pyogenes*, and *P. aeruginosa*. The antibiofilm efficacy was dose-dependent, with the most pronounced inhibition observed at concentrations corresponding to the minimum inhibitory concentration (MIC). This finding is particularly noteworthy given the clinical challenges posed by biofilm-associated infections, where conventional antibiotics often fail to achieve therapeutic efficacy [33, 34].

The differential antibiofilm activity observed between the two preparations may be influenced by the distinct phytochemical compositions resulting from the extraction process. MetE likely enriches bioactive compounds such as flavonoids and anthocyanins, which have been documented to disrupt biofilm formation and enhance microbial susceptibility [35, 36]. Additionally, the absence of antifungal activity in both forms suggests that the bioactive constituents of CM may exhibit selective antimicrobial properties, warranting further investigation into their mechanistic pathways.

Compared to existing literature, our study presents novel insights into the antimicrobial potential of Lyp CM, an area previously unexplored on a comprehensive scale. While prior studies have documented the antimicrobial properties of CM extracts, the direct comparison between Lyp and MetE forms provides a valuable perspective on the influence of processing methods on bioactivity [37, 38]. The high total anthocyanin content observed in our samples further underscores the potential therapeutic applications of CM, given the well-established antimicrobial properties of anthocyanins [39].

Despite the promising findings, certain limitations should be acknowledged. The study primarily focused on in vitro assessments, which may not fully capture the complexities of in vivo environments. Future research should explore the pharmacokinetics, toxicity, and potential synergistic effects of CM-derived compounds with conventional antibiotics. Additionally, elucidating the specific mechanisms underlying the observed antimicrobial and antibiofilm activities could inform the development of targeted therapeutic interventions.

The present study underscores the potential of Cornelian cherry, particularly in its MetE form, as a source of bioactive compounds with significant antimicrobial and antibiofilm properties. These findings contribute to the growing body of evidence supporting the exploration of plant-based antimicrobials as complementary or alternative strategies in combating antimicrobial resistance [22, 25]. Continued research in this domain is imperative to harness the full therapeutic potential of CM and to address the pressing global health challenge posed by antimicrobial-resistant infections.

Table 2. Inhibitory effects of Lyp and MetE forms of CM on biofilm formation of pathogenic microorganisms.

	Biofilm inhibition rate (%)					
	Lyp			MetE		
	Concentrations*			Concentrations*		
	MIC	1/2 MIC	1/4 MIC	MIC	1/2 MIC	1/4 MIC
S. aureus ATCC 43300	92.30±2.80	87.80±4.27	64.60±5.50	62.9±9.19	39.5±4.98	18.2±5.04
S. aureus ATCC 29213	60.5±0.52	64.3±1.29	63.8±1.96	40.9±0.60	52.7±4.78	47.6±8.36
S. aureus ATCC 25923	73.7±10.23	27.8±11.48	12.4±5.76	52.3±3.23	35.7±2.14	18.3±3.65
E. faecalis ATCC 29212	46.9±5.43	38.8±4.41	21.8±7.27	55.2±7.01	1.02±11.6	0.5±3.07
S. mutans ATCC 25175	17.5±7.77	14.6±3.27	0.2±9.42	11.5±0.91	12.8±2.32	20.1±3.12
S. pyogenes ATCC 19615	69.9±5.22	70.2±2.11	59.6±7.13	55.87±3.88	47.56±4.56	51.82±1.89
P. aeruginosa ATCC 27853	65.32±6.57	32.17±3.21	33.15±1.44	75.4±3.45	48.6±3.21	31.25±2.55
P. aeruginosa PAO1	61.89±2.87	41.25±0.94	12.91±1.24	64.89±3.44	51.44±2.89	11.25±3.25
K. pneumoniae ATCC 4352	18.25±1.25**	21.14±2.35	11.2±1.88	23.68±6.25	35.23±3.27	12.65±4.58
P. mirabilis ATCC 14153	12.8±1.39	19.2±2.87	14.25±1.12	19.89±1.88	12.58±3.25	20.8±2.95
S. typhimurium ATCC 14028	22.3±1.07	12.9±3.24	14.9±3.21	18.25±2.01	23.4±0.08	10.23±2.37
S. epidermidis ATCC 12228	28.25±3.74	12.28±1.02	20.58±1.68	25.8±12.31	30.7±11.14	25.1±8.52
A. baumannii ATCC 19606	67.27±6.41	50.34±5.28	31.66±2.69	84.8±3.84	66.9±0.39	28.9±5.77
E. coli ATCC 25922	27.32±1.25***	11.25±2.87	14,56±1.32	11.25±1.11	18.32±3.65	22.31±2.12

Lyp: Lyophilized, MetE: Methanol extract, MIC: Minimum inhibitory concentration, * To avoid antimicrobial activity, samples were tested at MIC and below MIC concentrations, ** Due to the absence of antimicrobial activity, testing was initiated at a concentration of 15.63 mg/ml, *** Due to the absence of antimicrobial activity, testing was initiated at a concentration of 1.96 mg/ml.

4. CONCLUSION

This study demonstrates the significant antimicrobial and antibiofilm activities of lyophilized Cornelian cherry (*Cornus mas* L.; CM) and its methanol extract against clinically important pathogens. The methanol extract exhibited superior antibacterial efficacy, inhibiting all tested bacterial strains, while the lyophilized form was ineffective against *K. pneumoniae* and *E. coli*. Both forms showed notable antibiofilm properties, particularly against *S. aureus*, *E. faecalis*, *S. pyogenes*, and *P. aeruginosa*, likely due to their high phenolic and anthocyanin content. The absence of antifungal activity indicates a selective antibacterial effect, more pronounced against Gram-positive bacteria. These findings highlight CM's potential as a natural antimicrobial agent; however, further in vivo studies and toxicity assessments are essential to fully explore its therapeutic applications.

5. MATERIALS AND METHODS

5.1 Plant Material and Lyophilization

In August - September 2020, cornelian cherry (*Cornus mas* L.; CM) grown in Erzurum, Uzundere Gölbaşı Neighborhood was purchased. Collected samples were identified by Dr. Gizem Emre from Marmara University Faculty of Pharmacy (Istanbul, Turkey), (Voucher Number: MARE-22458). Fresh CM fruits purchased for the study were mashed with a 32 mm pore pulper machine for seed removal; the outer skin of the CM was included in the puree. Afterwards, the Lyp drying process was carried out by sublimation with a G-Ray 125 freeze-dry machine.

5.2 Determination of total anthocyanin content

For the total anthocyanin analysis, samples were sent to Yeditepe University laboratory. The content of the extracted samples was determined according to the method reported by Giusti and Wrolstad (2001), that is based on the work of Fuleki and Francis (1986) [39, 40]. The total amount of monomeric anthocyanin was calculated in terms of cyanidin 3-glucoside.

5.3 Methanol Extraction

Maceration method was applied for the preparation of MetE. 20 g of Lyp CM product was weighed, and 100 ml of methanol was added. After 24 h, the methanol was filtered, and 100 ml of methanol was added to the remaining residue. The filtrate was continued for 3 days, and the methanol was evaporated in a rotary evaporator. To be used in the experiments, it was dissolved in Dimethyl sulfoxide.

5.4 Antimicrobial activity

Antimicrobial activity was determined by agar well diffusion method. Then, the minimum inhibitory concentration (MIC) and minimum bactericidal and fungicidal concentrations of the samples were determined by broth microdilution method against the microorganisms with activity.

5.4.1 Agar well diffusion method

The antimicrobial activity of the samples was determined for *P. aeruginosa* ATCC 27853, *P. aeruginosa* PAO1, *S. aureus* ATCC 43300, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 4352, *P. mirabilis* ATCC 14153, *S. typhimurium* ATCC 14028, *S. epidermidis* ATCC 12228, *A. baumannii* ATCC 19606, *E. faecalis* ATCC 29212, *S. mutans* ATCC 25175, *S. pyogenes* ATCC 19615, *E. coli* ATCC 25922 bacteria and *C. albicans* ATCC 90028, *C. albicans* ATCC 10231, *C. tropicalis* kuen 1021 and *C. glabrata* ATCC 90030 yeast strains. Microorganisms were obtained from the collection of the Department of Pharmaceutical Microbiology, Marmara University, Faculty of Pharmacy. Microorganism suspensions were prepared from colonies on solid media in 0.85% physiological saline solution (PSS) and adjusted to Mc Farland 0.5 standard turbidity with a concentration of 10^8 CFU/ml for bacterial suspensions and 10^6 CFU/ml for yeast suspensions. These suspensions were spread on the surface of Mueller Hinton agar for bacteria and Sabouraud dextrose agar for yeasts. Wells with a diameter of 5 mm were made on the medium using a sterile punch at certain intervals and 50 µl of the extract were placed in the wells. In addition, meropenem (10 µg/well) for bacteria and amphotericin B (100 µg/well) for yeasts were used as positive controls and DMSO and physiological saline were used as negative controls [41, 42].

5.4.2. Determination of minimum inhibitory concentration and minimum bactericidal concentration for bacteria

Minimum inhibitory concentration (MIC) was determined by broth microdilution method according to EUCAST standards. Bacterial suspensions were taken from 24 hours bacterial culture colonies, prepared according to Mc Farland 0.5 turbidity and diluted to 5 x 10^5 CFU/ml. Serial dilutions of the samples (range 100-0.049 mg/ml for Lyp and 250-0.122 mg/ml for MetE) were made in sterile U-bottom microdilution plates using Mueller Hinton broth medium (MHB). Then, 5µl of bacterial suspension was added to the wells containing the extract and incubated at 37 °C for 24 hours and the lowest sample concentrations without growth at the end of incubation were determined as minimum inhibitory concentration (MIC). MHB, DMSO, meropenem were used as control [43]. In the determination of minimum bactericidal concentration, 10 µl was taken from the wells where no growth was observed and inoculation to tryptic soy agar medium and incubated at 37 °C for 24 hours. After incubation, the lowest concentration at which no growth was observed was determined as the minimum bactericidal concentration (MBC).

5.5. Determination of antibiofilm activity of samples

P. aeruginosa ATCC 27853, *P. aeruginosa* PAO1, *S. aureus* ATCC 43300, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 4352, *P. mirabilis* ATCC 14153, *S. typhimurium* ATCC 14028, *S. epidermidis* ATCC 12228, *A. baumannii* ATCC 19606, *E. faecalis* ATCC 29212, *S. mutans* ATCC 25175, *S. pyogenes* ATCC 19615 and *E. coli* ATCC 25922 bacteria were inoculated in 5 ml Tryptic Soy broth- 1% glucose (TSB-glucose) and was incubated at 37°C for 24 hours (48 hours for *S. pyogenes* and *S. mutans*). Bacterial suspensions equivalent to McFarland 0.5 standard in TSB-glucose were prepared from the cultures. The prepared suspensions were diluted with TSB-glucose to a final concentration of 5×10^5 CFU/ml. The prepared bacterial suspensions were dispensed 180 µl into the wells of the flat bottom microplate. To avoid antimicrobial activity, samples were tested at MIC and below MIC concentrations. According to the MIC values of the samples against each strain tested, the final concentrations of the CM samples in the wells were prepared in TSB as 1, 1/2, and 1/4 times of the MIC value. Each sample was added as 20 µl to the respective wells containing bacteria in the plate. Since the antimicrobial activity of Lyp against *E. coli* and *K. pneumoniae* was not detected, the MIC values of MetE, which are 15.63 mg/ml for *E. coli* and 1.96 mg/ml for *K. pneumoniae*, were used for comparison with the antibiofilm activity of the MetE. The plates were incubated at 37 °C for 24 h. After incubation, the wells were carefully emptied with an automatic micropipette and washed twice with 250 µl PBS. After washing, 200 µl of 99% methanol was added to the wells and left for 15 minutes and the wells were emptied, and the microplate was left to dry. After the microplate was dried, 1% crystal violet (200 µl) was added to the wells and kept for 5 minutes, and the excess dye was removed by washing with water and the microplate was left to dry. 200 µl of 95% ethanol was added to the wells and kept for 30 minutes and optical density values were measured at 595 nm in a microplate reader at the end of the period. The experiments were carried out in 3 repeats and the arithmetic averages of the optical density (OD) values of each strain incubated with samples of different concentrations were taken. OD values of negative and positive control wells were measured according to the applied method and their mean values were calculated. The positive control OD value of each strain was accepted as 100% and the % value corresponding to the OD values of the wells containing different concentrations of sample was determined according to this value. The absorbance values of the wells containing the sample were subtracted from the absorbance value of the negative control [33]. The change in biofilm formation rates of strains in the presence of different concentrations of sample was calculated by the following formula [34].

Biofilm change rate = [OD]-D /[OD]-C × 100
[OD]-D= OD value of wells containing different concentrations of samples
[OD]-C = OD value of the positive control well

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