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ANTI-BIOFILM ACTIVITY OF BENZYL SUBSTITUTED HEXAHYDROIMIDAZO [1,5-*a*] PYRIDINIUM BROMIDE SALTS AGAINST SOME PATHOGENIC MICROORGANISMS

Nur Ceyhan-Guvensen[1,*], Hayati Turkmen[2]

[1]Mugla Sitki Kocman University, Faculty of Sciences, Department of Biology, 48170 Mugla, Turkey
[2]Ege University, Faculty of Sciences, Department of Chemistry, 35100 Izmir, Turkey

ABSTRACT

The principle aim of this study was to evaluate the anti-biofilm activity of newly synthesized hexahydroimidazo [1,5-*a*] pyridinium bromides with varying benzyl substituents to test as biofilm eradication agents against 10 opportunistic pathogens. To our knowledge this important aspect of their anti-biofilm and antioxidant activity has not yet been discussed in the literature. Anti-biofilm activity of benzyl substituted hexahydroimidazo[1,5-*a*]pyridinium bromide salts (B1-B9) was tested accoriding to microtitre plate method. Additionally, the best biofilm production of *E.coli* ATCC 8739 was monitoring with SEM (Scanning Electron Microscopy). The antioxidant activity of these salts were studied by the method of ABTS (2,2 azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation. In this study we have shown that these compounds possess excellent, broad spectrum anti-biofilm activity against opportunistic microorganisms: the bacteria *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Micrococcus luteus, Proteus vulgaris, Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae* and the yeasts *Candida albicans, Candida krusei.* All of these compounds inhibited bacterial and fungal biofilm formation at concentrations ranging from 0.78 to 400 µg/mL, 3.12 to 50 µg/mL, respectively (biofilm inhibitory concentration=BIC). Additionally, these compounds have been found to possess antioxidant potential with the highest inhibitory percentage of 92.4% using ABTS radical cation assay. The obtained results revealed that all the salts displayed anti-biofilm and antioxidant activity against the tested microorganisms, indicating that these salts could use in the design of new therapeutics and more effective antimicrobials for the control and prevention of biofilm infections caused by the spread of pathogenic microorganisms to the environment.

KEYWORDS:
Hexahydroimidazo [1,5-a] pyridinium bromides, imidazolinium salts, anti-biofilm activities, antioxidant activities, pathogenic microorganisms

INTRODUCTION

Opportunistic microorganisms are rapidly emerging as problematic pathogens in healthcare settings. Biofilm formation is an important reason for bacterial resistance to antimicrobials. A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and extracellular DNA [1]. The microorganisms can attach to diverse abiotic such as foreign body materials, oil pipelines, industrial hot tubs or biotic systems such as heart valves, teeth, mucosa and grow in highly stable communities [2-5]. Biofilm-associated microorganisms behave differently from planktonic (freely suspended) organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem [3].

Clinically, microbial biofilms are extremely problematic, and are implicated in implantable medical device-associated infections as well as many non-implant related chronic infections in humans [6-7], many of which are recalcitrant to standard antimicrobial regimens. Microbial biofilms, with their community defenses, are a ubiquitous feature of bacteria in nature and in many bacterial infections. Native valve endocarditis, cystic fibrosis bronchopneumonia, periodontitis, bladder infections, otitis media, musculoskeletal infections, necrotizing fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, meloidosis, chronic tonsillitis, rhinosinusitis, infectious kidney stones, chronic wounds, dental caries, and infections associated with foreign body material (contact lenses, urinary and venous catheters, mechanical heart valves, arteriovenous shunts, vascular grafts, peritoneal dialysis peritonitis, endotracheal tubes, stents, orthopedic devices, intrauterine device, other prostheses, etc.) are all examples of biofilm-related diseases [1, 7-10]. All these chronic infections are characterized by persisting inflammation and tissue damage [3, 11]. Biofilm formation has been proposed to be a key to the survival of pathogens in the context of infections [12, 13]. Chronic infections are infections that 1) persist in spite of antibiotic therapy, the innate and adaptive immune system

and inflammatory response of the host, and 2) in contrast to colonization, are characterized by immune response and persisting pathology [14]. Industrially, microbial biofilms are responsible for billions of dollars in lost productivity every year, occurring in almost every water-based process causing pipe blockage corrosion and contamination [15, 16].

The resident bacterial community in a biofilm has added defenses and multiple mechanisms for survival, such as defenses against phagocytosis, UV radiation, viral attack, shear stress, and dehydration, as well as against biocides, antibiotics, and host immunity. Biofilms have demonstrated the ability to persist in 100 to 1000 times the concentrations of antibiotics and biocides that can inhibit planktonic cells [17-20]. Antibiotic treatment is often unsuccessful when bacteria are organized in biofilms, because the latter give bacteria potent increased antibiotic resistance [7, 21]. These biofilm characteristics are the root of many persistent bacterial infections [15]. The biofilm allow adaptation to overcome multiple stresses and to survive most sequential therapies [10, 22, 23].

Current disinfection practices rely on convensional disinfectants that are typically biocidal or toxic to bacteria, but as noted above, these biocides are typically ineffective at controlling biofouling [7, 24, 25]. In this case, control of biofilm formation or maintenance must be synthesized new anti-biofilm compounds that have anti-biofilm activities [16, 24, 26, 27]. Imidazole derivatives has attracted more attention during recent years due to their biological activities. These compounds have been shown to possess antihelmintic, antifungal, antibacterial and anticancer activities [28]. Besides their biological actions, diazol(in)ium salts find wide applications in the preparation of N-heterocyclic carbenes (NHCs) and their metal complexes [29-31], some of which have attracted important attention as potential pharmaceuticals [31]. In addition, diazol(in)ium salts, as potential antiseptics/disinfectants, have been researched by some investigators [32-35].

The imidazolinium, benzimidazolium and tetrahydropyrimidinium salts and their metal complexes previously designed by Türkmen et al. (Fig. 1.) (1-9) had been shown to possess low toxicity. According to the data, LD_{50} values of all these compounds after oral application in mice in 24 h showed low toxicity according to EPA and WHO [39]. In this regard, the present study was carried out to examine theirs efficacy against biofilm formation, and ability to eradicate preformed biofilms. The tested bacteria and fungi in the work are important opportunistic human pathogens that causes several chronic organ infections especially in immuno-compromised patients and in elders [22, 36-38]. Much effort is now being invested to understand the anti-biofilm activity of these salts, with a view toward designing and/or improving new therapeutics and more effective antimicrobial agents against persistent biofilm infections by pathogens.

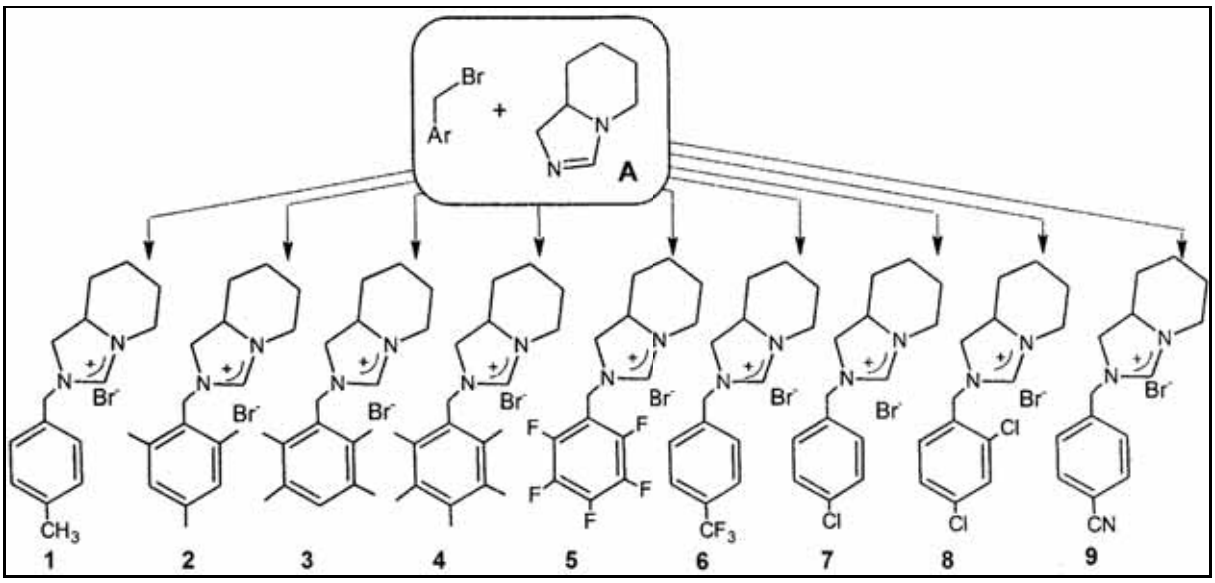


FIGURE 1
Synthesis and formula of the salts used for anti-biofilm activity. The purity of the salts was checked by TLC with Merck Kieselgel GF 254 Plates, elemental analyses and 1H and 13C NMR (Varian Mercury AS 400).

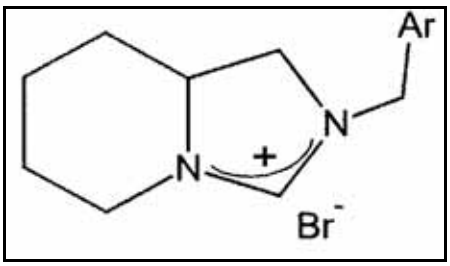


FIGURE 2
Structure of hexahydroimidazo [1,5-*a*] pyridinium bromide.

MATERIALS AND METHODS

Microbial Strains and Growth Media. In vitro antimicrobial studies were carried out against 10 test microorganisms (four Gram-positive bacteria: *S. aureus* (6538/P), *S. epidermidis* (ATCC 12228), *B. cereus* (CCM 99) and *M. luteus* (ATCC 9341), four Gram-negative bacteria: *P. vulgaris* (ATCC 6897), *E. coli* (ATCC 8739), *S. typhimurium* (CCM 5445) and *K. pneumoniae* (CCM 2318), and two yeasts: *C. albicans* (ATCC 10231) and *C. krusei* (ATCC 6258), which were obtained from the Microbiology Department Culture Collection of Ege University, Faculty of Science. Stock cultures of bacteria were maintained on nutrient agar (NA) (Merck) and yeasts were potato dextrose agar (PDA) (Merck) at 4 ºC.

Benzyl Substituted Hexahydroimidazo [1, 5-a] Pyridinium Bromide Salts. In our study the salts used for anti-biofilm activity had been previously synthesized by Türkmen et al. [39]. In their work, the salts had been obtained by quaternization of 1, 5, 6, 7, 8, 8a- hexahydroimidazo [1, 5- *a*] pyridine, by variously substituted benzyl bromides. 2- (2, 4, 6- tetramethylbenzyl)- 1, 5, 6, 7, 8, 8a- hexahydroimidazo [1,5- *a*] pyridin- 2-ium bromide (2), 2- (2, 3, 5, 6- tetramethylbenzyl)- 1, 5, 6, 7, 8,8ahexahydroimidazo[1, 5- *a*]pyridin- 2- ium bromide (3), 2-(pentamethylbenzyl)-1,5,6,7,8,8a-hexahydroimidazo[1,5-*a*]pyridin- 2 - ium bromide (4) are known in the literature [40, 41]. The other salts (1, 5-9) had been prepared according to the published procedure. The purity of the salts had been checked by TLC with Merck Kieselgel GF 254 Plates, elemental analyses and 1H and 13C NMR (Varian Mercury AS 400). Formula of the salts (1-9) were shown in Figure 1 and structure of hexahydroimidazo [1,5-*a*] pyridinium bromide was shown in Figure 2.

Anti-biofilm Activity Tests. Anti-biofilm activity of benzyl substituted hexahydroimidazo[1,5-*a*]pyridinium bromide salts (1-9) was tested in 96-well microtitre with glass pieces (1x1cm) [42-44]. Dilution series using sterile distilled water were prepared at the required quantities of 1600, 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/ml concentrations in wells and were added in TSB containing the bacterial suspension at 10^6 CFU/ml. The plates were incubated in the bacterial cultures for 24 h at 37 °C and in the fungal cultures for 72 h at 20 °C under stationary conditions and total growth was measured at 570 nm (Perkin Elmer-Lambda 25). Biofilm was measured by discarding the medium, rinsing the wells with 200 mL of water (three times) and staining the bound cells with crystal violet (0.4%, w/v) for 15 min, followed by washing with water (200 mL) to remove unbound stain. The dye was solubilized in 200 mL of 33% (v/v) acetic acid, and absorbance at 570 nm was determined using a microtitre plate reader (Thermo Scientific-Multiskan FC). Six replicates were used for each sample and each experiment was performed three times. The percentage of biofilm inhibition was calculated by the formula:
Percentage of inhibition = ([Control OD_{570nm} - Test OD_{570nm}]/Control OD_{570nm}) x 100

The biofilm inhibitory concentration (BIC) was determined as the lowest concentration that produced visible disruption of biofilm formation and a significant reduction in the readings when compared with the control wells at OD_{570nm} [45, 46]. The wells containing TSB and with the solutions of 1-9 were used as blanks.

SEM Analysis. The biofilm samples for SEM monitoring were prepared as described by Lembke et al. [47] and Thenmozhi et al. [43]. The biofilms on glass pieces (1x1cm) were fixed for 1 h in a solution containing 2.5% glutaraldehyde. The glass pieces were washed in 0.1M sodium acetate buffer (pH 7.3). Samples were dehydrated through a graded series of ethanol, critical-point dried, gold sputtered and examined with a JEOL, JSM-7600F SEM (USA).

Assay of Antioxidant Activities for 1-9 by In-vitro Method. The antioxidant activity was determined using ABTS (2,2 azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation method [48] with some modifications described by Rossini et al. [49] and Pieniz et al. [50]. ABTS radical cation was dissolved inwater (7 mM). ABTS radical cation was produced by reacting ABTS radical cation stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in dark at room temperature for 16 h before use. The stock solution was used for a maximum of 3 days. Before use, ABTS radical cation solution was diluted with ethanol, to an absorbance of 0.700 ± 0.020 at 734 nm. Samples were diluted with ethanol to obtain inhibition between 20 and 95% of the blank absorbance. Ascorbic acid was used as the standard in the range 0-9 µg/ml. After addition of 10 ml of sample (or standards) in

1.0 ml of ABTS radical cation solution, the absorbance was read at 30 s interval for 5 min. Likewise, a same proportion (10 ml) of culture medium or ultrapure water were used as controls. All determinations were carried out at least three times. The percentage inhibition of absorbance at 734 nm was calculated using ascorbic acid standard curve.

Statistical Analysis of Data. The data from all experiments were analysed using the SPSS software (version 16.0). Values were expressed as mean±SE. Standard errors (SE) between replicate samples for all experiments were calculated and an unpaired two-tailed t-test was performed to determine whether there was a statistically significant difference between growth as biofilms of microorganisms. All tests were considered significant at the level $p<0.05$. Error bar plots in figures are shown as one SE. The values for each experiment are the mean values of results from three experiments unless otherwise stated.

RESULTS AND DISCUSSION

The opportunistic pathogens are the leading cause of morbidity and mortality in enfected patients. Infections can be difficult to eradicate due to their propensity to form biofilms and their inherent resistance to antibiotics. Treatment regimens generally involve a rigorous and aggressive antibiotic assault to minimize the detrimental cycle of infection, inflammation, and subsequent scar tissue formation [37, 46]. Biofilm-embedded bacteria and yeast are tenacious and 100–1000 times more resistant to antimicrobial treatment [51]. Thus, inhibition of biofilm formation and/or dispersal of preformed biofilms may make biofilm-embedded bacteria and yeast more susceptible to antimicrobial agents. Hence, we have investigated the effects of variously substituted benzyl bromides (1-9) against on eight bacterial and two fungal opportunistic pathogens. To determine the BICs of the 1-9 on these microorganisms, the compounds with concentrations at 0.78 to 1600 µg/ml was assessed and the BICs were found as 0.78-400 µg/mL (Table 1).

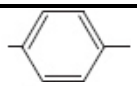
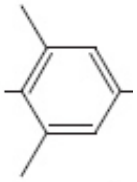
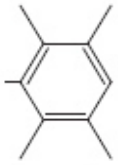
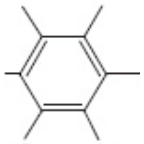
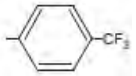
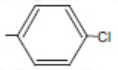
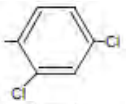
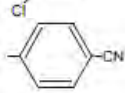
In the study, it was detected that was a poor correlation between lipophilicity of microbial cell walls and BIC values of multi methylated benzyl substituted salts, such as 1-4. The reasons of this might be high Clog*P* values (logarithms of 1-octanol/water partition coefficient parameter) [39] and lower anti-biofilm properties of 1-4. Also, steric and electronic interactions by influent. In addition to these, it was shown that anti-biofilm activity of electron-poor benzyls were higher than electron-rich ones in this work. Because most bacteria are negatively charged but still contain hydrophobic surface components, they prefer positive cations to bind in the biofilm [52, 53].

Moreover, the compounds tested here generally showed poor anti-biofilm activities against Gram-negative bacteria (Fig. 3.). The anti-biofilm activities against Gram-positive bacteria and fungus may depend on the differences between the cell structures of these microorganisms. Strong, tough, and elastic the Gram-negative cell wall is a remarkable structure which protects the contents of the cell. The cell walls of Gram-negative bacteria follow a more general structural format than that of gram-positive bacteria, which is strictly adhered to; Gram-negative bacteria have an outer membrane situated above a thin peptidoglycan layer. Sandwiched between the outer membrane and the plasma membrane, a concentrated gel-like matrix is found in the periplasmic space. Whereas, fungal cell wall has been shown to be primarily composed of chitin, glucans, mannans and glycoproteins [54].

The lowest BICs of antibiotics tested in the work were detected to be 12. 5 µg/mL against Gram-positive bacteria, Gram-negative and fungi (Table 1). Whereas, the BICs of the most active derivatives (6, 7, 8 and 9) were shown to be as low as 0.78 mg/mL. Interestingly, all these four derivates inhibited biofilm formation against two different yeast species at the same concentration 3.12 µg/ml (BIC). Additionally, they showed pronounced effect on the inhibition of Gram-positive and Gram-negative biofilm formation at very low concentrations (0.78-50 µg/mL). In Gram-negative bacteria, the outer membrane contains substautial amounts of protein and phospholipid and in addition, most or all of the lipopolysaccharide of the cell envelope. One of Gram-negative bacteria *K.pneumoniae* contains a discrete detectable layer of polysaccharides outside of the cell wall polymer named as capsule. Therefore, susceptibility of *K. pneumoniae* (CCM 2318) biofilm against the compounds might be lower than the other bacteria in the study (Fig. 3b).

The inhibition of biofilm formation was calculated for all tested microorganisms and their BICs were found to be 0.78-400 µg/mL which showed a significant reduction ($P< 0.05$) in biofilm formation by spectrophotometric quantification. In addition, visible reductions in biofilms of all tested microorganisms on the treated slides were observed by SEM monitoring. All data was not given in the study and *E. coli* was selected as a representating microorganism. Eventually, SEM analysis of the biofilms formed in the presence and absence of benzyl substituted hexahydroimidazo [1,5-a] pyridinium bromide salts confirmed the inhibition of *E.coli* biofilm formation by 1, 2, 3, 4, 6 and 7 (Fig. 4.). Combination of these active compounds with traditional antibiotics will help design more effective antimicrobials and therapeutic approaches for biofilm-treatments.

TABLE 1
Anti-biofilm and antioxidant activity of hexahydroimidazo [1,5-a] pyridinium bromides against tested pathogenic microorganisms.

Salts	Ar	Antioxidant activity (%)*	BIC (µg/ml)									
			Gram positive bacteria				Gram negative bacteria				Yeasts	
			SA	SE	BC	ML	PV	EC	ST	KP	CA	CK
1		75.5±0.09	25	25	100	50	50	25	50	25	12.5	12.5
2		12.5±1.00	100	50	400	50	200	400	200	400	50	25
3		15.8±2.06	100	25	400	50	25	100	100	400	50	12.5
4		56.2±0.71	50	25	200	25	50	50	50	50	50	6.25
5		60.8±0.47	25	25	200	50	100	100	100	50	50	3.12
6		92.4±1.82	12.5	3.12	6.25	3.12	12.5	3.12	3.12	50	3.12	3.12
7		90.5±2.00	12.5	3.12	6.25	3.12	25	12.5	6.25	50	3.12	3.12
8		89.6±0.15	3.12	3.12	25	6.25	12.5	12.5	3.12	25	3.12	3.12
9		87.5±0.20	12.5	12.5	25	6.25	25	12.5	0.78	50	3.12	3.12
Standard antibiotics	Ampicillin		50	12.5	100	6.25	25	25	12.5	50	-	-
	Nystatin		-	-	-	-	-	-	-	-	12.5	12.5

*: Values represent the mean±SE of three independent experiments; -: Not tested; BIC: Biofilm inhibitory concentration; SA: *Staphylococcus aureus;* SE: *Staphylococcus epidermidis;* BC: *Bacillus cereus;* ML: *Micrococcus luteus;* PV: *Proteus vulgaris;* EC: *Escherichia coli;* ST*: Salmonella typhimurium;* KP: *Klebsiella pneumoniae;* CA: *Candida albicans; and* CK: *Candida krusei.*

The antioxidant activity was evaluated by ABTS radical cation method (Table 1). 1-9 exhibited high ability to scavenge the radical ABTS, with inhibitory percentages ranging from 12.5 to 92.4% (Table 1). From the results of antioxidant activity, it was recognized clearly that 6 and 7 could produce the highest amount of antioxidant activity, 92.4% and 90.5%, respectively. Conversely, 2 and 3 showed weak antioxidant activity, with an inhibitory percentage of 12.5% and 15.8%, respectively. As a general result, there were noticeable antioxidant activities of these derivates. Considering their potency as potential antioxidants especially 6 and 7 can find wide application in functional pharmaceutical formulations.

CONCLUSION

A biofilm focus also provides new strategies for treatment of chronic infections. Biofilm-based treatments might block initial bacterial attachment to a surface, block or destroy EPS formation, inter-

fere with cell-cell signaling pathways, and use bacteriostatic or bactericidal agents at the same time. Microbial biofilms, which characterized by their resistance to the traditional antimicrobials, are considered as a renewable source of contamination by pathogens.

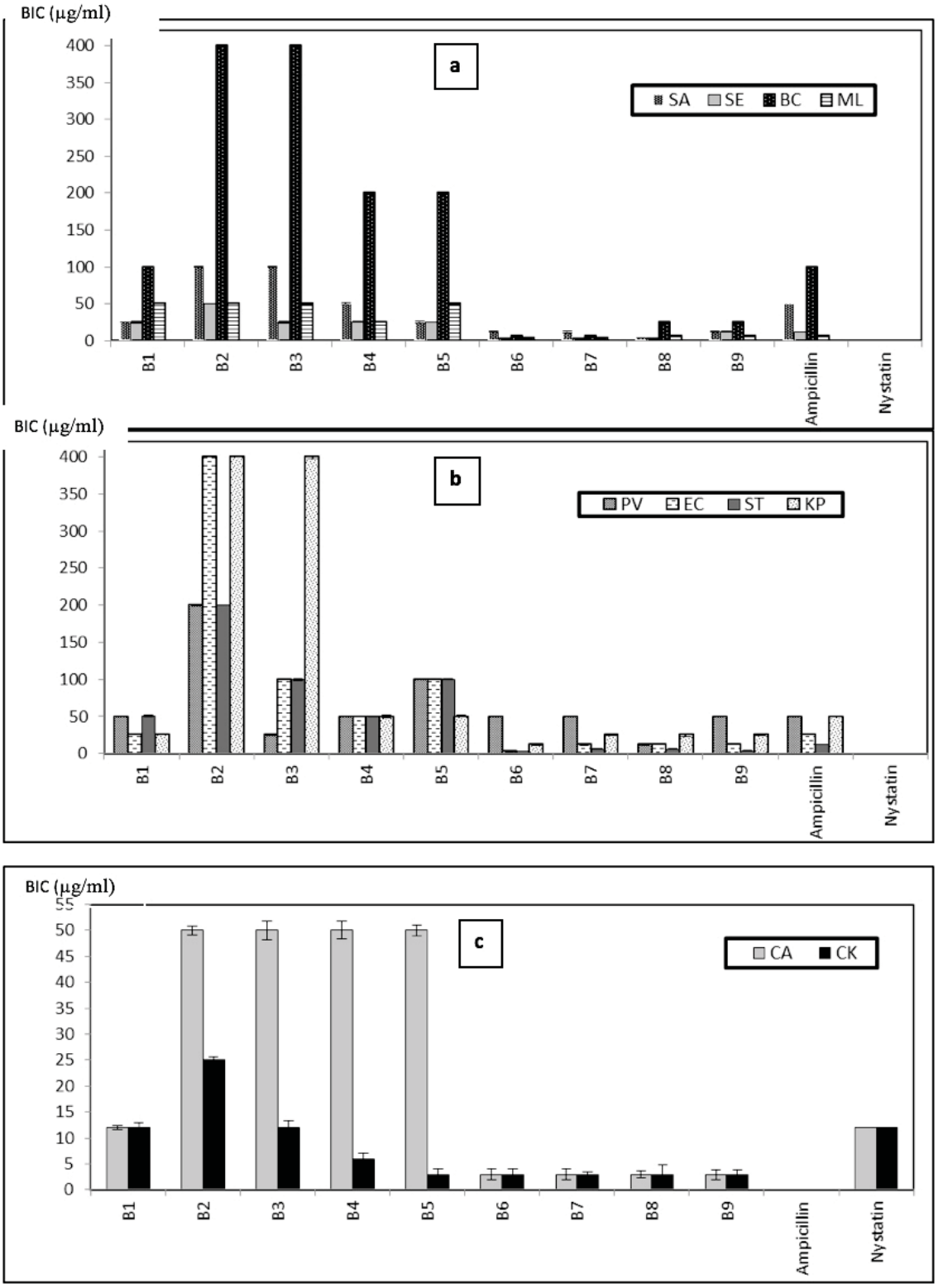


FIGURE 3
Inhibition effect of hexahydroimidazo[1,5-a]pyridinium bromides (1-9) and standard antibiotics (a) on Gram-positive bacteria, (b) on Gram-negative bacteria and (c) on yeasts.

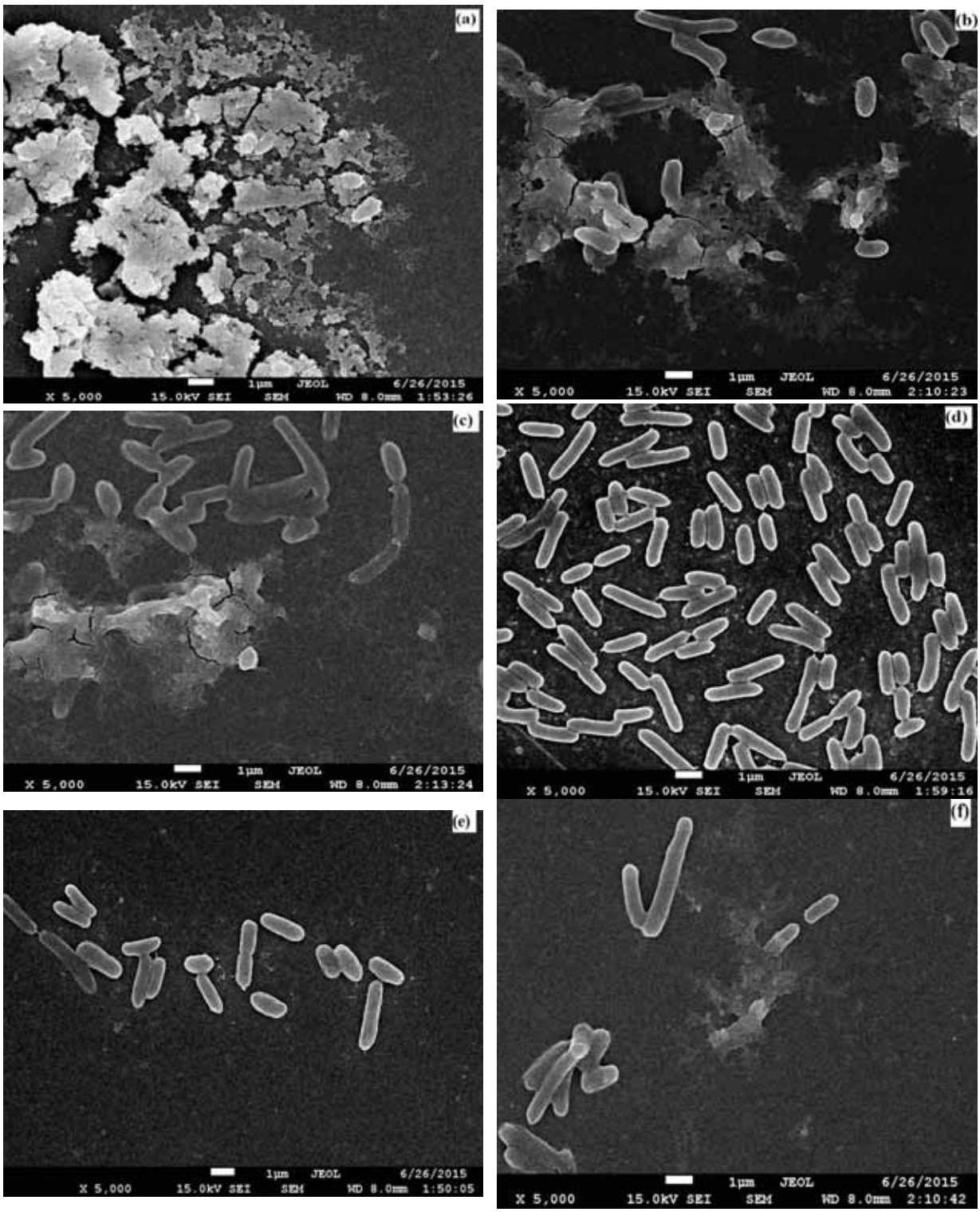


FIGURE 4
SEM images of effect of some hexahydroimidazo [1,5-a] pyridinium bromides on *E.coli* (ATCC 8739) biofilm. (a) biofilm formation by *E.coli* (derivate-free control), (b) biofilm treated with 2, (c) biofilm treated with 3, (d) biofilm sample treated with 4, (e) biofilm treated with 6, (f) biofilm treated with 7; scale bar:1 µm

Anti-biofilm activity-based theraphies that not only attempt to eradicate bacteria but also affect the biofilm's community structure and communications may prove more effective than a single or sequential strategy such as antibiotic therapy. Furthermore, the employment of anti-biofilm agents may help

address the significant patient cost (in terms of morbidity and mortality) and financial burden imposed by infections caused by persistent biofilm-microorganisms.

For these reasons, our research focus has been on anti-biofilm activity of benzyl substituted hexahydroimidazo[1,5-a] pyridinium bromides. The results show that tested microorganisms was susceptible in biofilm forms. They indicate that the imidazolinium, benzimidazolium and tetrahydropyrimidinium salts (1-9) and their metal complexes possess remarkable anti-biofilm activity and are effective against biofilm populations (sessile) of tested microorganisms. In this work, 1-9 shows superior characters as anti-biofilm effect and antioxidant property. Therefore, these groups may be new sources of anti-biofilm agents and antioxidants with potential value for therapeutics. They should be explored further as a means to control biofilm-associated infections caused by pathogens.

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The authors declare no conflict of interest.

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CORRESPONDING AUTHOR

Nur Ceyhan-Guvensen
Mugla Sitki Kocman University
Faculty of Sciences
Department of Biology
48170, Mugla – Turkey

e-mail: nurceyhan@msn.com