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RESEARCH / ARAŞTIRMA

Evaluation of Antimicrobial and Antibiofilm Effects of Kojic Acid Derivatives against Bacteria and Fungi

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ABSTRACT

Objective: In this study, it was aimed to investigate the antibacterial and antifungal activities of kojic acid derivatives against Gram-negative bacteria *(Pseudomonas aeruginosa and Escherichia coli*), Gram-positive bacteria *(Enterococcus faecalis and Staphylococcus aureus)* and fungal strain *(Candida albicans).*

Material and Methods: Antimicrobial activity profiles of the compounds were investigated by microdilution and disk diffusion method. Antibiofilm activities were investigated by spectrophotometric microplate method including crystal violet staining. Moreover, the impact of the compounds on both biofilm and cells was also clarified via scanning electron microscopy (SEM).

Results: Minimum inhibitory concentration values were found between 16 µg/mL to 1024 µg/mL. The most potent substance, 3b bearing 3,4-dichlorobenzylpiperazine moiety, has been shown to have inhibitory effects at 16-64 µg/mL concentrations. It was determined that 3a and 3b had stronger inhibitory effects than the other substances against *C. albicans*. In addition, antibacterial activity was discovered to be greater against Gram-positive strains than Gram-negative. It was also shown by SEM that compound 3b caused significant deformation on the cell wall and membrane of *E.* faecalis strain and inhibited the biofilm structures of *S. aureus* and *E. coli strains.*

Conclusion: Kojic acid derivatives were found to inhibit the growth of both bacterial and fungal pathogens at low concentrations. It was also shown that kojic acid derivatives were able to decrease biofilm formation. It is thought that, this will be a favourable scenario for novel therapeutic candidates since more novel compounds will be created, and the inhibitory mechanism will be explored in various research including phenotypic and genotypic experiments.

Keywords: Kojic acid derivatives, antimicrobial activity, antibiofilm activity, scanning electron microscopy.

Kojik Asit Türevlerinin Bakteri ve Mantarlara Karşı Antimikrobiyal ve Antibiyofilm Etkilerinin Değerlendirilmesi

ÖZET

Amaç: Bu çalışmada kojik asit türevlerinin Gram-negatif bakterilere *(Pseudomonas aeruginosa ve Escherichia coli),* Gram-pozitif bakterilere *(Enterococcus faecalis ve Staphylococcus aureus)* ve mantar kökenine *(Candida albicans)* karşı antibakteriyel ve antifungal aktivitelerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Bileşiklerin antimikrobiyal aktivite profilleri mikrodilüsyon ve disk difüzyon yöntemleri ile araştırıldı. Antibiyofilm aktiviteler kristal viyole ile boyamayı içeren spektrofotometrik mikroplaka yöntemi ile araştırıldı. Ayrıca bileşiklerin hem biyofilm hem de hücreler üzerindeki etkisi taramalı elektron mikroskobu (SEM) ile açıklığa kavuşturuldu.

Bulgular: Minimum inhibitör konsantrasyon değerleri 16 µg/mL ile 1024 µg/mL arasında bulundu. En güçlü madde olan, 3,4-diklorobenzilpiperazin kısmını taşıyan 3b'nin 16-64 µg/mL konsantrasyonlarda inhibitör etkiye sahip olduğu gösterildi. 3a ve 3b'nin *C. albicans'a* karşı diğer bileşiklere göre daha güçlü inhibitör etkileri olduğu saptandı. Ek olarak, antibakteriyel aktivitenin Gram-pozitif suşlara karşı, Gram-negatif suşlara göre daha fazla olduğu belirlendi. Ayrıca bileşik 3b'nin, SEM ile *E. faecalis* suşunun hücre duvarı ve membranında önemli deformasyona neden olduğu ve *S. aureus* ve *E. coli* suşlarının biyofilm yapılarını inhibe ettiği gösterildi.

Sonuç: Kojik asit türevlerinin düşük konsantrasyonlarda hem bakteriyel hem de fungal patojenlerin büyümesini engellediği belirlenmiştir. Ayrıca kojik asit türevlerinin biyofilm oluşumunu azaltabildiği gösterilmiştir. Bu, daha fazla yeni bileşik yaratılacağı ve engelleyici mekanizmanın fenotipik ve genotipik deneyler de dahil olmak üzere çeşitli araştırmalarda keşfedileceği için yeni terapötik adaylar için olumlu bir senaryo olacaktır.

Anahtar Kelimeler: Kojik asit türevleri, antimikrobiyal aktivite, antibiyofilm aktivite, taramalı elektron mikroskobu.

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If previously presented as a paper, paper type, place and date: The article has not been presented at any scientific event.

1. Introduction

Kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, KA) is a widely recognized fungal metabolite that was first identified from the mycelium of *Aspergillus oryzae* cultured on steamed rice (1). Since its discovery, KA has attracted considerable attention due to its flexible chemical structure enabling many molecular modifications. Therefore, a considerable number of derivatives have been synthesized and found applications in various fields, including pharmaceutical, food, agricultural, and cosmetic industries.

Biofilms are clusters of bacteria that are embedded in a protective matrix and attached to abiotic surfaces (2). Bacteria on biofilm structures show up to 1000-fold enhanced antibiotic resistance to the broad spectrum of antimicrobial drugs, and they are shielded from human immune responses, environmental factors, and antimicrobial agents (3). Biofilm structures seem to point to the origin of the problem and they are formed in response to chronic infections (4). As a result, the number of biofilm/bacterial infections that result in morbidity and mortality is increasing. Therefore, it is essential to discover new antibiofilm agents. Our research group's synthetic Mannich bases derived from KA have been shown to possess a variety of pharmacological activities, including anticonvulsant and antibacterial (5,6), antiviral (7), anticancer (8–10), antioxidant (11) and antityrosinase (12). The general structures of the compounds tested in the present work are given in Fig. 1.

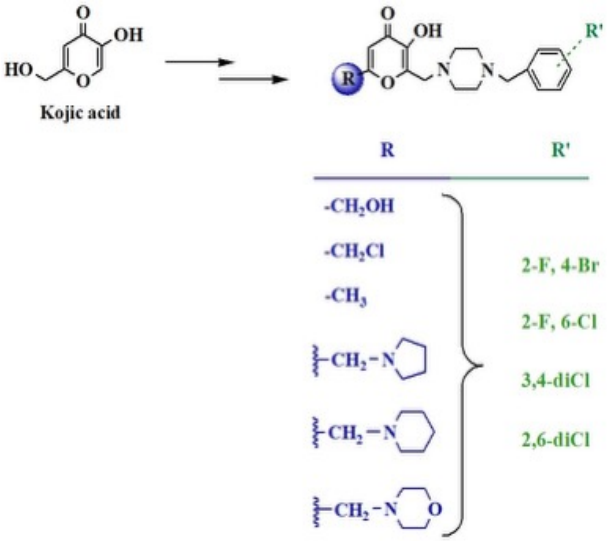


Figure 1. Chemical structures of tested Mannich bases as antimicrobial and antibiofilm agents.

Since some of the antifungal agents (e.g. itraconazole, ketoconazole, posaconazole) and antibiotics (linezolid) that are currently used for the treatment of infections contain an azole and/or a piperazine ring in their structures, Mannich bases of KA analogues were designed with this perspective. In an early study, antifungal effects of allomaltol (5-hydroxy-2-methyl-4H-pyran-4-one) derivatives, which is obtained from the reduction of chlorokojic acid (5-hydroxy-2-chloromethyl-4H-pyran-4-one), against *C. albicans, Candida krusei and Candida parapsilosis* were evaluated. When compared to the reference antifungal agent fluconazole against *C. krusei*, compounds with phenylpiperazine moieties and halogen atoms shown notable antifungal activity against *Candida* species with equal minimum inhibitory concentration (MIC) values (5). Afterwards, new chlorokojic acid Mannich bases were synthesized and tested for their antiviral and antibacterial properties. The antibacterial activity of some compounds was proven antibacterial activity against *Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumonia* and *Escherichia coli* strains. Additionally, the antiviral effects against RNA virus (Human parainfluenza virus type 3) and DNA virus (Herpes simplex virus type 1) were evaluated. A derivative was found as potent as the reference compound acyclovir, against herpes simplex virus type 1 (7). In studies carried out in order to explore the effects of Mannich bases on pathogenic microorganisms in more detail, their antitubercular activity and antidermatophyte activities were also investigated (6,13).

In a study, it was investigated that the antibacterial and antifungal activities of KA and kojyl carbamates of different drugs, and the kojyl carbamate of rimantadine was found to have the strongest activity against fungi and bacteria (14). The MIC values were between 31-1250 µg/mL against S. aureus, 125-625 µg/mL against *C. albicans, E. coli and P. aeruginosa*. In another study, the antimicrobial activity of the isoxazole conjugates bearing kojic acid moiety was investigated, and the MIC values were between 12.5-100 µg/mL concentrations against *S. aureus* and *Candida kefyr,* the MIC values were 100 µg/mL against *E. coli* (15).

The antimicrobial potential of the KA formulations including nanoemulsions, liposomes, nanoparticles, magnetic nanocomposites and biodegradable films has been evaluated against microorganisms in different studies (16–20). Azhar et al. (16) investigated the antibacterial effects of KA ester-based nanoemulsion and KA ester against *S. aureus*, and reported that the nanoemulsion formulation of KA was found to be more effective than the KA ester. Ezzat et al. (17) investigated the antibacterial effects of gelatinized core liposomes including KA, and they showed that the liposome formulations exhibited equal MIC to the free KA against *S. aureus*. In another study, it was reported that the chitosan tri-polyphosphate nanoparticles and magnetic nanoparticles including KA was enhanced the present antibacterial activity against *P. aeruginosa* and *S. aureus* (18). In addition, composite biodegradable films including KA were also investigated to determine antibacterial activity for packaging materials, and the films showed antibacterial effectiveness (20).

Besides, some Mannich bases of KA including 3,4-dichloro and 2,6-dichloro benzylpiperazine substituent were patented which are namely compounds 3a and 4a in the present work (Table 1), for their anti-mycobacterial, antidermatophytic, antityrosinase, antioxidant and antiaging activities as "Kojic Acid-Derived Mannich Bases with Biological Effect" title (11). Especially compound carrying 2,6-dichloro benzylpiperazine structure was shown to have a MIC values of 1 µg/mL against the three dermatophyte strains: *Microsporum gypseum, Epidermophyton floccosum* and *Trichophyton mentagrophytes* var. *erinacei*. Based on these findings, we decided to focus on halogen-substituted benzylpiperazine derivatives for additional experiments.

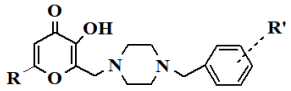
Based on the known potent antimicrobial effect in the bioisosteres of this class of compounds with previously proven cytotoxic activities, we aimed to investigate the antimicrobial and antibiofilm activities of the halogen-substituted benzylpiperazine derivatives against fungi and bacteria in the present study. To the best of our knowledge, this is the first study that investigates the antibiofilm activities of the halogen-substituted benzylpiperazine derivatives against fungal and Gram-negative/Gram-positive bacterial strains by spectrophotometric and microscopic experiments.

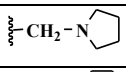
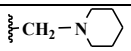
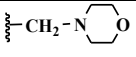
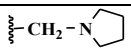
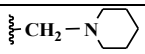
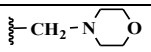
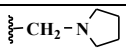
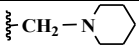
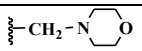
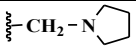
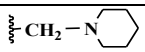
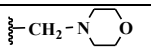
2. Materials and Methods

2.1. Chemistry

The synthetic routes to isosteric KA analogues and their Mannich bases are depicted in Fig. 4, starting from KA. The synthesis and chemical identification of all the compounds were reported in our previous studies (6–13,21). In a preceding study, KA derivatives including compound 3b were synthesized and screened for its antimicrobial activities against fungi and bacteria (7). Similar to the mentioned study, we investigated the

Table 1. Chemical structure, inhibition zone diameter (mm ± SD) and minimum inhibitory concentration values (µg/mL) of the compounds.



Compound/ Method	R	R'	Gram-positive bacteria *S. aureus*	Gram-positive bacteria *E. faecalis*	Gram-negative bacteria *E. coli*	Gram-negative bacteria *P. aeruginosa*	Fungi (Yeast) *C. albicans*
1a[a]/DD	-CH_2OH	2-F, 4-Br	-	-	-	-	-
1a[a]/MD			128	128	512	256	256
1b[b]/DD	-CH_2Cl	2-F, 4-Br	13.67±0.58	-	-	-	-
1b[b]/MD			128	128	256	256	>1024
1c[c]/DD	-CH_3	2-F, 4-Br	-	-	9.00±0.00	7.67±0.58	-
1c[c]/MD			>1024	512	512	512	256
1d[b]/DD		2-F, 4-Br	8.00±0.00	13.00±0.00	13.00±0.00	8.00±0.00	-
1d[b]/MD			>1024	256	256	256	128
1e[b]/DD		2-F, 4-Br	11.67±0.58	-	9.00±0.00	8.00±0.00	-
1e[b]/MD			512	512	512	512	>1024
1f[b]/DD		2-F, 4-Br	8.00±0.00	12.67±0.58	-	-	-
1f[b]/MD			>1024	512	256	512	256
2a[a]/DD	-CH_2OH	2-F, 6-Cl	8.00±0.00	-	9.00±0.00	-	-
2a[a]/MD			512	512	512	256	512
2b[b]/DD	-CH_2Cl	2-F, 6-Cl	-	13.00±0.00	10.00±0.00	9.00±0.00	7.67±0.58
2b[b]/MD			64	256	64	256	>1024
2c[c]/DD	-CH_3	2-F, 6-Cl	7.67±0.58	-	11.00±0.00	8.00±0.00	-
2c[c]/MD			>1024	1024	256	256	256
2d[a]/DD		2-F, 6-Cl	8.00±0.00	12.00±0.00	9.00±0.00	7.67±0.58	-
2d[a]/MD			64	32	256	256	128
2e[a]/DD		2-F, 6-Cl	7.67±0.58	8.00±0.00	8.00±0.00	-	-
2e[a]/MD			>1024	1024	256	128	512
2f[a]/DD		2-F, 6-Cl	8.00±0.00	13.00±0.00	8.00±0.00	8.00±0.00	-
2f[a]/MD			>1024	1024	512	256	256
3a[d]/DD	-CH_2OH	3,4-diCl	7.67±0.58	-	12.00±0.00	8.67±0.58	17.00±0.00
3a[d]/MD			256	1024	512	256	256
3b[e]/DD	-CH_2Cl	3,4-diCl	13.67±0.58	-	11.00±0.00	9.00±0.00	-
3b[e]/MD			16	16	32	64	16
3c[b]/DD	-CH_3	3,4-diCl	9.00±0.00	16.67±0.58	11.00±0.00	9.00±0.00	-
3c[b]/MD			>1024	1024	512	256	128
3d[b]/DD		3,4-diCl	10.00±0.00	10.00±0.00	10.67±0.58	10.00±0.00	9.00±0.00
3d[b]/MD			256	128	128	256	>1024
3e[b]/DD		3,4-diCl	10.00±0.00	14.00±0.00	10.67±0.58	10.00±0.00	-
3e[b]/MD			512	512	128	256	64
3f[f]/DD		3,4-diCl	8.00±0.00	9.00±0.00	11.00±0.00	10.00±0.00	9.00±0.00
3f[f]/MD			512	256	512	256	128
4a[d]/DD	-CH_2OH	2,6-diCl	-	-	-	-	-
4a[d]/MD			128	64	256	256	256
4b[b]/DD	-CH_2Cl	2,6-diCl	-	-	13.00±0.00	9.00±0.00	-
4b[b]/MD			128	256	128	512	>1024
4c[c]/DD	-CH_3	2,6-diCl	7.33±0.58	-	7.67±0.58	-	-
4c[c]/MD			512	1024	512	256	512
4d[a]/DD		2,6-diCl	8.00±0.00	-	9.00±0.00	9.00±0.00	-
4d[a]/MD			256	128	512	256	256
4e[a]/DD		2,6-diCl	7.33±0.58	-	8.00±0.00	9.00±0.00	-
4e[a]/MD			256	256	512	256	>1024
4f[a]/DD		2,6-diCl	8.00±0.00	13.00±0.00	-	-	-
4f[a]/MD			512	128	512	256	>1024
KA/DD			17.33±0.58	14.00±0.00	11.67±0.58	9.33±0.58	14.33±0.58
KA/MD			256	512	1024	1024	1024
CIP/DD			25.00±0.00	24.00±0.00	32.00±0.00	26.00±0.00	nt
CIP/MD			0.5	1.0	0.01	1	nt
FLU/DD			nt	nt	nt	Nt	32.00±0.00
FLU/MD			nt	nt	nt	Nt	1
DMSO/DD			-	-	-	-	-
DMSO/MD			-	-	-	-	-

DD: Disk diffusion test, MD: Microdilution method, (-): no effect, nt: not tested, CIP: Ciprofloxacin, FLU: Fluconazole, KA: Kojic acid, a: Karakaya et al., 2019, b: Karakaya et al., 2018, c: Ercan et al., 2020, d: Aytemir et al., 2018, e: Aytemir and Özçelik, 2010, f: Karakaya et al., 2019b.

antimicrobial and antibiofilm activities of compound 3b against *E. faecalis* ATCC 29212 strain in order to determine the structure-activity relationship among a larger number of KA derivatives. Twenty-four compounds were dissolved in dimethyl sulfoxide (DMSO) and sterile distilled water (1:1), and stock solutions of the compounds were sterilized using membrane filters (0.22 µg pore-size) (Millipore).

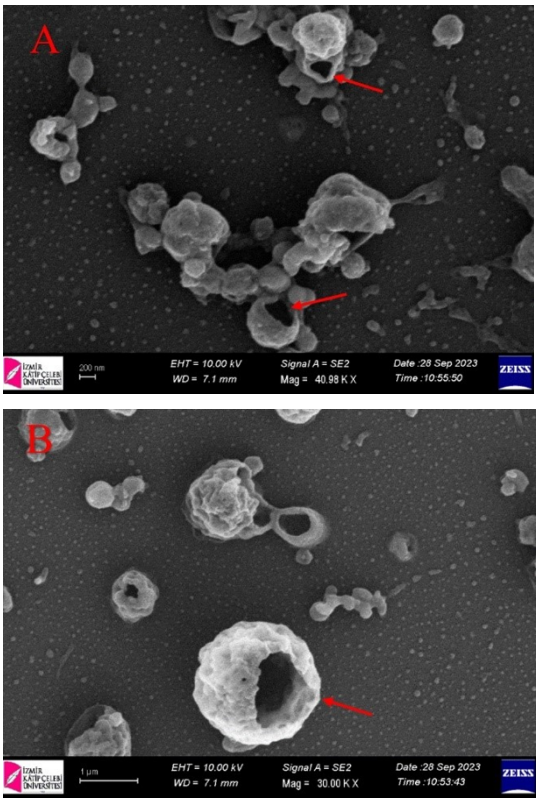


Figure 4. (A-B) The cell damage of E. faecalis strain after exposure to the compound is indicated by the arrows.

2.2. Microorganisms

The antimicrobial activity tests for the synthesized compounds were applied by using American-type culture collection (ATCC) strains. *Candida albicans* ATCC 90028, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were used in the antibacterial and antifungal activity experiments including twenty-four compounds. All of the strains were kept at -80 °C in brain-heart infusion broth (Merck) with 10% glycerol. The bacterial and yeast strains were cultured on Mueller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA) (Oxoid), respectively, prior to the antimicrobial activity assays.

2.3. Antibacterial and Antifungal Activity Experiments

2.3.1. Disk Diffusion Test

A disk diffusion test was performed to determine the inhibition zone diameters (22). Fresh bacterial and fungal colonies were collected using sterile swabs, and suspensions were prepared with sterile physiological saline. The turbidity of the microorganism suspensions was adjusted to 0.5 McFarland standard using a densitometer (Biosan, DEN-1). These suspensions were spread onto agar plates using sterile cotton swabs. Sterile blank disks (6 mm) (Oxoid) were placed on the plates, and 10 µL of the test compounds were applied to the disks. MHA plates were incubated at 37 °C for 24 hours for bacteria, while SDA plates were incubated for 48 hours for yeast. Ciprofloxacin and fluconazole were used as reference drugs, and DMSO was tested separately. Inhibition zone diameters were measured after the incubation period. The test was repeated three times, and the results were evaluated.

2.3.2. Microdilution Method

The minimum inhibitory concentration (MIC) values of the compounds were evaluated by broth microdilution method with minor modification (23). Fungal and bacterial suspensions were prepared with fresh colonies and physiological saline, and a densitometer was used to adjust to 0.5 McFarland turbidity. The bacterial suspensions were diluted 10-2 and yeast suspensions were diluted 10-1. Sterile 96-well microplate wells were filled with 50 µL of Sabouraud dextrose broth (SDB) (Merck) for yeast and Mueller-Hinton II broth (cation-adjusted) (Merck) for bacteria. The compounds (50 µL) were added to the first wells of the microplates, and serial dilutions (1/2) were completed. After adding 50 µL of bacterial and fungal suspensions to the wells, the microplates were incubated at 37 °C for 24 hours for bacterial strains and 48 hours for yeast strains. Fluconazole and ciprofloxacin studied as reference antimicrobial agents, and quality control ranges were evaluated according to the EUCAST criteria (Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. Version 13.0, 2023 and Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts, EUCAST Defin. Doc. E. Def 7.3.1, 2017). All samples were tested in three replicates. The minimum inhibitory concentration (MIC) values that prevented the growth of bacteria and fungi were identified.

2.4. Antibiofilm Activity Experiments

Antibiofilm activities of the compounds at different concentrations (10, 100 and 1000 µg/mL) were investigated by a spectrophotometric microplate method (24). The effects of the compounds on both biofilm production and mature biofilm structure of fungal and bacterial strains were investigated. Firstly, fungal and bacterial strains were grown on SDA and MHA, respectively. Compound solution (20 µL) was added into the wells of 96-well microplates that include 160 µL tryptic soy broth (TSB) (Merck) containing 2.5 % glucose. Then, the microbial suspensions (20 µL) (0.5 McFarland turbidity) were inoculated to the wells of microplates. The microplates were incubated for 24 hours at 37 °C. After the incubation period, the contents of each well were aspirated, and 200 µL of phosphate buffered saline (PBS) was used to wash each well three times. Following the washing steps, the microplates were kept for air drying. Then, 200 µL of methanol was added to the wells for 15 min to fix the bacterial and fungal cells that adhered to the well surface. After emptying the wells and discarding the methanol, the wells were allowed to air dry. Following this step, 0.1% crystal violet solution (200 µL) was applied to each well and incubated at room temperature for 5 minutes. The wells were washed three times with tap water, crystal violet solution was removed from the wells and the microplates were air dried. 200 µL of 95% ethanol was added to each well for 15 mins. Following that, the contents of each well were transferred to sterile 96-well microplates for performing the measurements. The spectrophotometric measurements were performed at 570 nm using a microplate reader (BMG-Labtech, Clariostar). Inhibitory effects on biofilm production were evaluated by measuring the optical density (O.D.) values of the wells.

The antibiofilm activity of the compounds on mature biofilms of bacterial and fungal strains were also investigated (24). TSB (160 µL) containing 2.5 % glucose and microbial suspensions (20 µL, 0.5 McFarland turbidity) were incubated in 96-well microplates at 37 °C for 24 h in order to mature biofilm before exposure to the compounds. The compound solutions at appropriate final concentrations (1000, 100 and 10 µg/mL) were added to the microplate wells. The microplates were re-incubated for 24 hours at 37 °C. Afterwards, the crystal violet

staining assay described above was performed and the antibiofilm activity was evaluated by measuring the O.D. values of the wells. As negative control groups, the O.D. values of the samples that contained media without an inoculum were used. The O.D. values of the samples that include only TSB and the microorganism suspension, without test compound, were used as the biofilm control group. Three standard deviations over the mean O.D. of the negative controls was the definition of the cut-off O.D. (O.D.c). All tests were conducted in triplicate. Statistical analyses were performed using GraphPad Prism 5.03 program (t-test, Duncan's mean comparison test), with $p \leq 0.05$ considered statistically significant. The inhibition/induction impact ratio of the compounds on biofilm formation and mature biofilm was calculated according to the formula given below:

Impact ratio on biofilm formation (IRBF) (%) = $(O.D._A - O.D._B) / O.D._A \times 100$

($O.D._A$: The optical density of biofilm control well, $O.D._B$: The optical density of the well that contain the test compound).

2.5. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was employed to observe the antibiofilm activity against *S. aureus, E. faecalis* and *E. coli* strains. To perform this, strains were first inoculated onto tryptic soy agar (TSA, Merck) and allowed to incubate for overnight. Fresh colonies were employed to create bacterial suspensions with a turbidity of 0.5 McFarland in PBS, which were then utilised in tests to produce a biofilm (25). Six well-clear, polystyrene, sterile, flat-bottomed cell culture plates were used for biofilm research by SEM. Each well was cautiously filled with spherical coverslips of sixteen millimetres in diameter and varying in thickness from 0.13 to 0.16 mm (Marienfeld). Tryptic soy broth (TSB, Merck) was added to the wells at a volume of 1800 µL with the addition of 0.25 % glucose. Subsequently, 200 µL freshly made bacterial suspensions were introduced into every well. Antibiofilm activity of the compounds was observed by adding the compound 100 µg/mL final concentration, and the control wells received no addition of compound. Then, plates were incubated for 24 hours at 37 °C. Following incubation, three PBS washing were performed on the biofilm-forming surfaces to eliminate any remaining media and non-biofilm-forming cells. Then, the samples were placed in wells with 2.5 % glutaraldehyde solution and allowed to biofilm fix for two h. Coverslips were cleaned of glutaraldehyde by rinsing them three times in PBS. In the end, samples were dehydrated in each solution for 20 min using ethanol percentages that increased (35 %, 50 %, 75 %, 90 %, 95 % and 99 %). Each coverslip was allowed to air dry at 30 °C for 18 h. Each sample was gold sputter-coated (Quorum Q150 Res) during the drying step, and pictures were taken with the SEM device (Carl Zeiss 300VP) (26,27).

2.6. Ethical Aspect of the Research

Ethical permission is not required for this research, which was conducted in an in vitro experimental environment using standard microorganisms under laboratory conditions.

3. Results

3.1. Disk Diffusion Test

The mean inhibitory zone diameters for twenty-four compounds against C. albicans, P. aeruginosa, E. coli, E. faecalis and S. aureus were determined by disk diffusion test. Mean inhibition zone diameter values and standard deviation (SD) values in fungi and bacteria were presented in Table 1. Based on the findings, inhibition zone diameters were found to be in the following ranges: 7.67-17.00 mm for C. albicans, 7.67-10.00 mm for P. aeruginosa, 7.67-13.00 mm for E. coli, 7.33-13.67 mm for S. aureus and 8.00-16.67 mm for E. faecalis. It was discovered that the compounds 1b and 3b had the largest inhibition zone diameters against S. aureus and the compound 3c had the largest inhibition zone diameters against E. faecalis. Furthermore, 3a exhibited the largest inhibition zone diameters against C. albicans. DMSO had no inhibitory effects on microbial growth at studied concentrations, and the inhibition zone diameter values of fluconazole and ciprofloxacin were found to be within quality control ranges based on The European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria.

3.2. Microdilution Method

The MIC values of the compounds against *C. albicans, P. aeruginosa, E. coli, E. faecalis* and *S. aureus* were demonstrated in Table 1 according to the data of microdilution method. The following ranges of 16-512 µg/mL for E. coli, 16->1024 µg/mL for S. aureus, 64-512 µg/mL for P. aeruginosa, 32-1024 µg/mL for E. faecalis and 16->1024 µg/mL for *C. albicans* were found to be the MIC values of the compounds against fungal and bacterial strains. It was discovered that 3b had stronger inhibitory effects than the other substances against *C. albicans, E. coli, P. aeruginosa, S. aureus* and *E. faecalis*. In addition, the growth of E. faecalis strain was inhibited in the presence of 3b at 16 µg/mL and 1d compounds at 32 µg/mL concentrations. DMSO had no inhibitory effects against microorganisms at studied concentrations, and MIC values of ciprofloxacin and fluconazole were found to be within quality control limits according to the EUCAST.

3.3. Antibiofilm Activity

In this study, the antibiofilm effects of the 3a and 3b against fungi and bacteria were summarized in Table 2-3. The alteration on mature biofilm and biofilm production of microorganisms were presented with % inhibition/induction rates and p values. According to the results, the compound 3a inhibited the biofilm production of *E. coli* and *P. aeruginosa* strains. The effects of compound 3a on biofilm formation levels of the bacterial and fungal strains are summarized in Table 3. No statistically significant inhibitory effect of 3a at high concentrations was detected on biofilm production levels of *S. aureus.* On the other hand, biofilm production levels of *E. coli* was reduced more than 50% even in the presence of the compound at the lowest concentration (10 µg/mL). According to the findings of studies on mature biofilm structures, 3a had an antibiofilm effect *on E. coli* and *P. aeruginosa*, while no significant effect was observed on *C. albicans* and *S. aureus.*

In this study, compound 3b was found to have an inhibitory effect on *S. aureus, E. faecalis, E. coli* and *P. aeruginosa* biofilm production levels. Remarkably, even in the presence of the compound 3b at a low concentration (10 µg/mL), biofilm production levels of *E. coli* was suppressed. When compound 3b was present at a lower dosage (10 µg/mL), it had no discernible influence on the mature biofilm structure of the strains whereas the compound 3b at high concentration (1000 µg/mL) demonstrated inhibitory effects on the mature biofilm structures of *E. faecalis, E. coli* and *P. aeruginosa*. In contrast to the inhibition at higher concentrations, it was also found that the biofilm production levels of E. faecalis increased in the presence of 3b at 100 µg/mL concentration. Additionally, the mature biofilm and biofilm production levels of *C. albicans* were not significantly affected by compound 3b.

3.4. SEM Analyses

Confirmation of the biofilm removal capacity of the compound 3b (100 µg/mL) was determined by SEM experiments. According to the results, the biofilm structures of *E. coli* and *S. aureus* were significantly reduced after exposure to the compound (Figure 2).

The morphological alteration on the cells of *E. faecalis* strain after exposure to compound 3b was another outcome of the SEM studies (Figure 3). It has been noted that when the substance is applied, there may be a significant degree of cell deformation. This implies that the antibacterial activity of 3b compound may arise as a result of interaction with the cell wall and cell membrane.

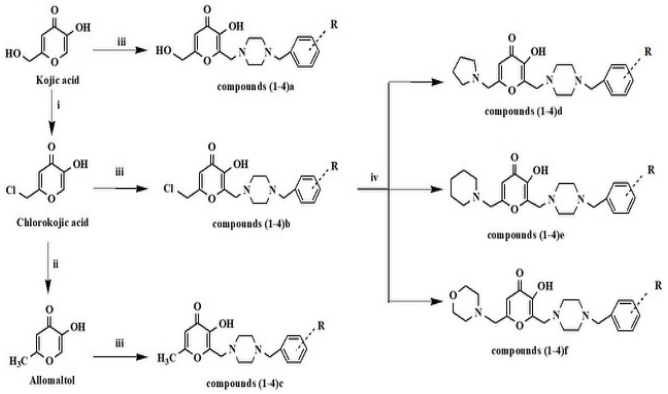
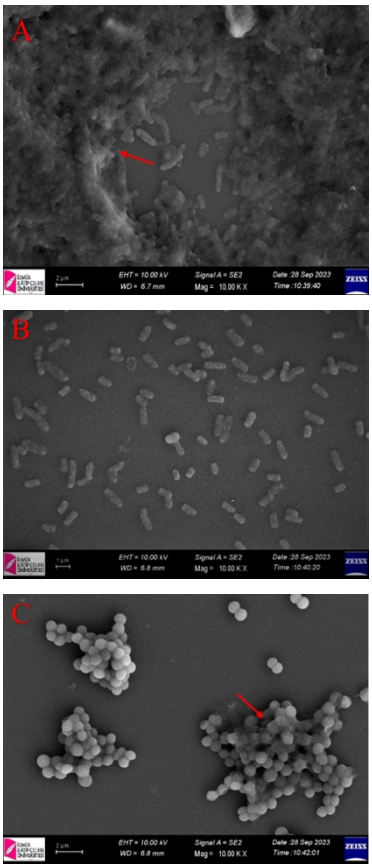


Figure 2. Synthesis of Mannich bases; Reagents and conditions: (i) SOCl2; (ii) Zn/HCl; (iii) halogen-substituted benzyl piperazine derivatives, formaline 37%, MeOH, rt; (iv) pyrrolidine /piperidine/morpholine, K2CO3, DMF, 0ºC (9).



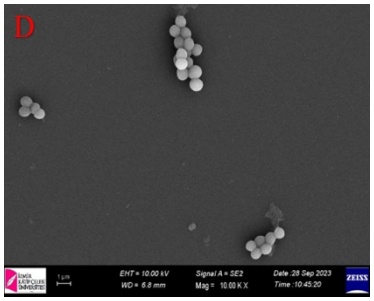


Figure 3. (A) Biofilm structure of E. coli strain, (B) E. coli cells without biofilm structure, (C) Biofilm structure of S. aureus strain, (D) S. aureus cells without biofilm structure. The matrix in which the bacteria are embedded is indicated by the arrows in the pictures where biofilm formation is observed. Arrows were not provided in cases when biofilm was not seen.

Table 2. The effects of the compound 3a on biofilm production and mature biofilm of bacterial and fungal strains (% inhibition/induction and p values).

Strains	Effect on Biofilm Production			Effect on Mature Biofilm		
	1000 µg/mL	100 µg/mL	10 µg/mL	1000 µg/mL	100 µg/mL	10 µg/mL
S. aureus	-	-	61.2% ↑ 0.0002*	-	-	-
E. faecalis	60.5% ↓ 0.0004*	-	27.4% ↓ 0.03*	41.4% ↓ 0.0009*	-	-
E. coli	62.2% ↓ 0.0002*	60.4% ↓ 0.0008*	63.2% ↓ 0.0005*	56.1% ↓ 0.0001*	20.7% ↓ 0.002*	-
P. aeruginosa	80.1% ↓ 0.0007*	21.2% ↑ 0.0006*	-	59.0% ↓ <0.0001*	24.3% ↓ 0.0015*	24.7% ↓ 0.001*
C. albicans	72.9% ↓ 0.0001*	66.3%↑ 0.0003*	80.6% ↑ 0.0002*	-	-	-

(-): No effect on biofilm, (↓): Inhibition of biofilm, (↑): Induction of biofilm,
*: Statistically significant.

Table 3. The effects of the compound 3b on biofilm production and mature biofilm of bacterial and fungal strains (% inhibition/induction and p values).

Strains	Effect on Biofilm Production			Effect on Mature Biofilm		
	1000 µg/mL	100 µg/mL	10 µg/mL	1000 µg/mL	100 µg/mL	10 µg/mL
S. aureus	77.0% ↓ 0.023*	67.6% ↓ <0.0001*	49.6% ↓ 0.08	-	15.0% ↓ 0.007*	-
E. faecalis	46.4% ↓ 0.01*	22.7% ↑ 0.002*	-	40.1% ↓ 0.0004*	-	-
E. coli	89.1% ↓ 0.04*	74.4% ↓ <0.0001*	89.6% ↓ 0.017*	35.8% ↓ 0.003*	18.3% ↓ 0.001*	-
P. aeruginosa	81.3% ↓ <0.0001*	48.4% ↓ 0.03*	-	40.6% ↓ 0.0004*	10.8% ↓ 0.015*	-
C. albicans	-	-	-	-	-	-

(-): No effect on biofilm, (↓): Inhibition of biofilm, (↑): Induction of biofilm,
*: Statistically significant.

4. Discussion

The advent of antibiotics has significantly transformed the course of medicine. A significant number of antibacterial molecules have been approved and placed into use since the middle of the 20th century and saved the lives of millions (28). This breakthrough has also contributed to the development of modern medicine, enabling many medical techniques to be done without the risk of infection (29). However, the phenomenon of resistance has significantly increased (30). In such a situation, it becomes essential to investigate novel options for therapy or assistance before resistance depletes the available therapeutic possibilities.

Since therapeutically viable dosages of antimicrobial agents do not affect the bacteria associated with biofilm structures, anti-biofilm therapies often target the suppression of biofilm development (31). To achieve this goal, we investigated the prevention of bacterial adherence to surfaces and the prevention of biofilm formation.

In this study, the antimicrobial activities of halogen-substituted benzylpiperazine derivatives of KA were investigated in terms of their chemical modifications, divided into the following four groups, each containing six compounds: 2-F,4-Br group, 2-F,6-Br group, 3,4-diCl group, 2,6-diCl group. The MIC values of the compounds in 2-F,4-Br group ranged from 128 µg/mL to 1024 µg/mL according to the broth microdilution test results. The compounds 1a and 1b showed the strong antimicrobial effect in this group with the MIC value of 128 µg/mL against Gram-positive bacteria. It was determined that the compound with the highest antifungal activity in 2-F,4-Br group was 1d with 128 µg/mL MIC value against *C. albicans*. The MIC values of the compounds (2a-2f) also referred to as 2-F,6-Cl group, were detected within the range of 32->1024 µg/mL. Among the compounds in this group, 2d showed a higher antibacterial effect against Gram-positive bacteria than the other compounds. The MIC values of 2d against *S. aureus* and *E. faecalis* strains were detected as 64 µg/mL and 32 µg/mL, respectively. Additionally, it was also sighted that 2d is the most active derivative against fungi among this series. The MIC values of 1d and 2d were detected as 128 µg/mL and this value was 8-fold lower than MIC value of KA against *C. albicans*. For the compounds in 3,4-diCl group and 2,6-diCl group, it was determined that the MIC values ranged from 16 to 1024 µg/mL. It was evident that the compounds in 3,4-diCl group showed stronger antifungal activity than 2,6-diCl group. Especially it was detected by microdilution method that the compounds 3b and 3e were the most active within 3,4-diCl group against *C. albicans*.

In a previous study, the antimicrobial activity of chlorokojic acid derivatives was investigated, and the MIC values of the compounds ranged d between8-128 µg/mL against *S. aureus* and *E. coli* and 16-128 µg/mL against *E. faecalis* and *P. aeruginosa*, and 8-32 µg/mL against *C. albicans* (6). In another study, chlorokojic acid derivatives including compound 3b were synthesized and screened for their antimicrobial activities, and the MIC values were determined against standard strains and clinical isolates of *S. aureus* (MIC: 1-128 µg/mL), *E. faecalis* (MIC: 8-128 µg/mL), *E. coli* (4-64 µg/mL), *P. aeruginosa* (MIC: 2-64 µg/mL) and *C. albicans* (4-16 µg/mL) (7). In this study, we investigated the antimicrobial and antibiofilm activities of compound 3b against the same *E. faecalis* ATCC 29212 strain in the mentioned study in order to determine the structure-effect relationship of KA derivatives, and MIC of the compound was found to be 16 µg/mL, same as the value found in the previous study.

In addition to the microdilution method, the disk diffusion test results were also evaluated in this study, and the most effective compounds were found to be 1b and 3b (Inhibition zone diameter: 13.67 mm) against S. aureus. Additionally, compound 3c had the highest inhibition zone diameter (16.67 mm) against *E. faecalis* strain. The highest inhibition zone diameters (13 mm) were determined with 1d and 4b compounds against *E. coli*. 3d, 3e and 3f were more effective than the other compounds against P. aeruginosa, exhibiting inhibition zone diameters of 10 mm. Interestingly, 3a was found to be the most effective compound as evidenced by an inhibition zone diameter of 17 mm *against C. albicans*. We believe that the differences in antimicrobial activity observed in the results of the microdilution method and disk diffusion test were due to variations in the solubility features of the compounds in agar and broth media used in the experiments.

An additional noteworthy observation upon examining all series is that the derivatives exhibiting the lowest MIC values across all series correspond to molecules where -CH2Cl is situated at position 2 within the molecule. Wu et al. (32) demonstrated the critical role that the free -CH2OH group at position 2 of the typical KA molecule plays a significant role in membrane leakage and damage. However, it is observed that groups with -CH2Cl instead of -CH2OH group in the relevant position are more active in the derivatives included in this study. The achievement of -Cl substitution at this position is believed to enhance its toxicity, potentially through increased membrane interaction.

When comparing the antimicrobial effects of the compounds, 3b was one of the most prominent compound among all halogen-substituted benzylpiperazine derivatives tested in this study, with its potent antibiofilm, antibacterial and antifungal effects. The most effective derivative against both Gram-positive and Gram-negative bacteria was the compound 3b, according to a comparison of the synthesized compounds within MIC ranges. It was found that the MIC values of 3b against *E. faecalis* and *P. aeruginosa* were 32-fold lower than the MIC values of KA, as the MIC values of compound 3b against *S. aureus* and *E. coli* were detected to be 16-fold lower than the MIC values of KA.

In the literature, studies investigating the effects of KA on biofilm production and/or mature biofilm of different microorganisms are limited. In addition to antibacterial activity experiments, the antibiofilm activity of 3a and 3b was also investigated in this study. The effects of the compounds on biofilm production levels and mature biofilms of microorganisms were evaluated by the spectrophotometric microplate method. Upon analysing the data from antibiofilm activity assays, it was noted that the inhibitory effects of 3b on biofilm production of the strains were more potent than the effects on mature biofilm structure. High concentrations of the compound 3b (1000 µg/mL) decreased the ability of *E. coli*, *S. aureus, P. aeruginosa* and *E. faecalis* strains to produce biofilm at the rates of 77.0%, 46.4%, 89.1% and 81.3%, respectively. It has been found that the 3b compound has a stronger inhibitory effect on the biofilm production of Gram-negative bacteria when compared to Gram-positive bacteria. Unlike antifungal potential, no significant effect was observed on the biofilm formation or mature biofilm structure of *C. albicans* in the presence of 3b. One of the remarkable findings of this study is that 3b showed a strong antibiofilm effect, especially on the tested Gram-negative bacteria. In addition, 3a inhibited the mature biofilm and biofilm production levels of *E. coli, P. aeruginosa* and *E. faecalis* at 1000 µg/mL concentrations. Biofilm production levels of *C. albicans* were also inhibited in the presence of 3a at 1000 µg/mL level. Interestingly, biofilm production levels of C. albicans strain were induced in the presence of the compound 3a at 10 and 100 µg/mL as the biofilm production levels were induced in *S. aureus* and *P. aeruginosa* in the presence of 3a at 10 and 100 µg/mL concentrations, respectively. In a study, the bioactivities of KA against biofilm and bacterial growth were investigated, and it was reported that the KA had weak antibacterial property (MIC: 24 mM), as the biofilm vitality and biomass of Acinetobacter baumannii strain significantly decreased at 6 mM (33). It was also reported that, a wide range of mechanisms, including stress

responses, metabolic and transcriptional alterations on bacterial virulence factors, cell communication systems (Quorum sensing and secretion), energy and substance metabolisms, may be involved in the regulation of biofilm structure in *A. baumannii* (33). More efforts are required to investigate the mechanism of action of the compounds on biofilm structures of microorganisms. Targeting of biofilm-related genes including quorum sensing genes may be a reasonable strategy for the inhibition of biofilm production and mature biofilm in microorganisms.

The crystal violet method is often favoured as a dependable technique for biofilm mass detection. Nevertheless, one drawback of this approach is that the crystal violet dye employed stains both viable and non-viable cells (34). Despite enabling the detection of a reduction in total biofilm mass, this method does not indicate cell viability (35). Therefore, in addition to the crystal violet results, SEM microscopy was also investigated. Upon completion of these experiments, it is shown that untreated *E. coli* cells form dense biofilms and become embedded in the matrix. However, when treated with 3b cells, the quantity of bacteria is drastically decreased, and the structure of the biofilm is eliminated. Comparatively, it was found that the biofilm structure was clearly evident, and the *S. aureus* cells attached in those that did not interact with 3b. It was observed that the number of bacteria and biofilm reduced in the 3b treated condition. On the other hand, although no significant difference was observed in the biofilm production levels of the *E. faecalis* strain compared to the negative control group, the cell damage was clearly demonstrated by SEM. Another intriguing finding thus far is the morphological alteration on the *E. faecalis cells*, which was unexpected based on the antibiofilm activity observed with crystal violet data at the studied dosages. This demonstrates how the compound 3b affects the cell membrane and the cell wall, leading to severe deformity and perforations. The significance of this information in elucidating the structure-effect relationship unique to *E. faecalis* species has been established.

5. Conclusion and Recommendations

In this study, the antimicrobial activities of halogen-substituted benzylpiperazine derivatives of KA were investigated by disk diffusion and microdilution methods. The MICs of the compounds were found to vary between 16 µg/mL to 1024 µg/mL. The most active compound bearing 3,4-dichlorobenzylpiperazine moiety and -CH2Cl group, has shown to have inhibitory effects at 16-64 µg/mL. The compound 3b had stronger inhibitory effects than the other substances against *C. albicans* as 3a exhibited the largest inhibition zone diameters against this pathogen. In addition, the activity was discovered to be greater against Gram-positive bacteria than Gram-negative strains. It was also shown by SEM that the compound 3b caused significant deformation on the cell wall and membrane of *E. faecalis* strain and inhibited the biofilm structures of *S. aureus* and *E. coli* strains. The antibiofilm activity of 3a and 3b was also investigated by the spectrophotometric microplate method. It was noted that 3b had more inhibitory effects on the strains' ability to produce biofilm than it did on the structure of mature biofilm. According to the findings of SEM analyses, 3b caused severe deformation and perforations by affecting the cell wall and membrane. Based on the results of this study, it is believed that further studies on the potential antimicrobial and antibiofilm effects of halogen-substituted benzylpiperazine derivatives of KA against pathogen microorganisms will be beneficial for the development of new bioactive agents and for the discovery of potential effects against clinical isolates. We also believe that investigating the combined effects of the KA derivatives and antimicrobial drugs against clinical isolates of fungi and bacteria is crucial, and further experiments may provide new treatment opportunities against infections.

6. Contribution to the Field

Kojic acid derivatives were found to inhibit the growth of both bacterial and fungal pathogens at low concentrations. In addition to the antimicrobial effects, kojic acid derivatives were able to decrease biofilm formation, which is one of the main virulence factors for infections. Biofilm production levels *of E. coli* and *S. aureus* were reduced after exposure to the compound 3b as observed through scanning electron microscopy. Compound 3b affects the cell membrane and the cell wall of *E. faecalis*, leading to severe deformity and perforations on the cell. As a result, we believe that the data obtained in this study will contribute to the field of infection control and therapy.

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None

Conflict of interest
The authors report no conflicts of interest.

Authorship Contribution

Concept: İÖ, GK, Hİ, MDA; Design: İÖ, GK, Hİ, MDA; Supervision: İÖ, GK, YT, MDA; Funding: İÖ, GK, YT, Hİ, MDA; Materials: İÖ, YT, AT; Data Collection/Processing: İÖ, GK, YT, AT; Analysis/Interpretation: İÖ, GK, YT, AT; Literature Review: İÖ, GK, YT; Manuscript Writing: İÖ, GK, YT, AT, Hİ, MDA; Critical Review: İÖ, GK, YT, MDA

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