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Antimicrobial and Antibiofilm Activity of 4-Benzylidene-2-methyl-oxazoline-5-one against Pathogen Bacteria

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ABSTRACT

One of the major challenges in healthcare is the rise of antibiotic resistance, where bacteria have developed resistance to a wide range of commonly available antibiotics. These resilient bacteria pose a significant threat to public health, leading to severe illnesses and creating a substantial challenge for treatment. Therefore, the discovery of new antimicrobial agents is crucial in controlling the spread of infections caused by drug-resistant bacteria. This study focuses on the synthesis of oxazoline and investigates the antimicrobial and anti-biofilm properties of this compound named 4-benzylidene-2-methyl-oxazoline-5-one. The structure of the oxazoline compound was precisely characterized by ^{1}H NMR, ^{13}C NMR, and FT-IR. The antibacterial activity was assessed on *S. aureus* using the agar-well diffusion while the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined to identify the concentration ranges with significant inhibitory effects. *S. aureus* is one of the most noticeable microorganisms in medical and clinical sciences, especially nosocomial infections. Additionally, the study evaluated the compound's impact on biofilm formation and the expression of the *icaA* gene. The results from the MIC and MBC testing demonstrated that the compound exhibits both bacteriostatic and bactericidal effects on both Gram-positive and Gram-negative bacteria, as well as yeast. Furthermore, the presence of this antibacterial compound led to a reduction in *icaA* gene expression. 4-Benzylidene-2-methyl-oxazoline-5-one displayed significant antimicrobial activity and hindered biofilm formation. Moreover, it was observed that 4-benzylidene-2-methyl-oxazoline-5-one induced cell death through its toxic effects on MCF-7 cells. When it was tested at concentrations of 0.01, 0.1, and 1 mg/ml this compound exhibited cytotoxic effects and significantly decreased cell viability.

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Introduction

Biofilms are densely packed microbial cells that grow on some surfaces and are primarily enclosed in a polysaccharide matrix. Biofilms can develop on diverse surfaces, such as biological tissues, implanted medical devices, piping systems in industrial or drinking water setups, or natural aquatic environments (Flemming and Wuertz, 2019). Bacterial biofilms are now obviously recognized as a major cause of human diseases (Alexandra *et al*., 2013; Dobretsov *et al*., 2013) such as refractory chronic diseases including cystic fibrosis, chronic wounds, endocarditis, cystitis, and infections caused by indwelling medical devices (Gallego-Hernandez *et al*., 2020).

Hospital infections often occur due to biofilms formed by microorganisms, particularly on medical instruments such as catheters (central venous, urinary), prosthetic heart valves, respirators, and orthopedic devices (Stewart and Bjarnsholt, 2020).

Under unfavorable external conditions, biofilm bacteria can adapt themselves to inconvenient conditions such as antibiotics and toxic compounds. One of the most important reasons for the increased resistance is the penetration barrier that biofilms may present to antimicrobials. As reported, many virulence factors are involved in *Staphylococcus aureus* pathogenicity, such as extracellular toxins and cell surface structures that aid in colonization and detrimental function in tissues, as well as immune evasion (Otto, 2014). It has been demonstrated that among all of the pathogenic activities of *staphylococci*, one of the best-understood mechanisms involved in the development of biofilms is *polysaccharide*-mediated, termed *polysaccharide* intercellular adhesion (PIA) or polymeric *N-acetyl-glucosamine* (PNAG), which is produced via enzymes encoded by the *ica* operon- (Torlak *et al*., 2017).

Researchers have recently tried to synthesize new antimicrobial drugs against antibiotic-resistant bacteria. Heterocyclic compounds, particularly those containing nitrogen, sulfur, and oxygen, have garnered significant attention in the field of medicinal chemistry due to their pharmacological activities (Al-Mulla, 2017). In recent years, derivatives of oxazoles (1,3-oxazole) and isoxazoles (1,2-oxazole) have emerged as crucial pharmacophores, playing a pivotal role in diverse biological and medicinal activities. One of the most significant heterocyclic compounds is Oxazol-5-one, which possesses an oxazole ring containing one nitrogen atom and one oxygen atom. It serves as the fundamental structure for numerous pharmaceuticals. These compounds exhibit a remarkable ability to engage with various enzymes and receptors present in biological systems, thereby demonstrating a broad range of biological activities such as antimicrobial (Sedenkova *et al.,* 2022), anticancer (Fawzi *et al.,* 2023), antifungal (Trefzger *et al.,* 2020), antitumor (Wang *et al.,* 2023), insecticidal (Huang *et al.,* 2022), anti-inflammatory (Mota *et al.,* 2019), and antituberculosis properties (Abhale *et al.,* 2017). Furthermore, it has been observed that various functional groups with substitutions in the C-2 or C-4 positions have a significant impact on these activities. Specifically, the presence of a substituted (p-nitro) exocyclic phenyl group at the C-4 position of the oxazolone moiety has been shown to have a remarkable effect on immunosuppressive activity (Tandel and Mammen, 2008). Similarly, the substitution of an amine group at the C-4 position in isoxazolone (Cycloserine) has been shown to influence its antibiotic activity (Prosser and de Carvalho, 2013). In the case of 4-Benzylidene-2-methyl-oxazoline-5-one, one of its oxazolone derivatives, there is a methyl group located at the C-2 position (Fig. 1).

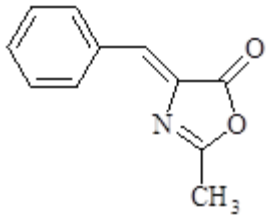

Fig. 1. The 4-Benzylidene-2-methyl-oxazoline-5-one (BMO)

Given the significance of biofilm formation by pathogenic bacteria, our study aims to investigate the antibacterial and anti-biofilm formation properties of 4-Benzylidene-2-methyl-oxazoline-5-one (BMO) on *S. aureus*. *S. aureus* is a microorganism of considerable importance in medical and clinical sciences, particularly in the context of nosocomial infections. Consequently, our study focuses on evaluating the impact of this compound on the expression of the *icaA* gene in *S. aureus*.

Materials and methods

Synthesis of 4-benzylidene-2-methyl-oxazoline-5-one

N-acetylglycine was prepared by adding acetic anhydride 95% (100 ml) to 30 ml of aqueous glycine solution with vigorous stirring for 20 min (Herbst and Shemin, 1939). The product was filtered and dried at 100° C to form a white crystal. In a 250 ml flask, N-acetylglycine (4.00 g, 0.03 mM) was mixed with freshly distilled

benzaldehyde (5.40 g, 0.05 mM) as well as anhydrous sodium acetate (2.05 g, 0.02 mM). Acetic anhydride (9.16 g, 0.09 mM) was added, and the flask was warmed in the steam bath with continuous stirring for 20 min (until the solution was completely prepared). The solution was refluxed for 1 h and cooled. Then, the yellow-formed crystals were transferred to a Buchner funnel and washed thoroughly with cold water and ether. After being dried in a vacuum desiccator over phosphorus pentoxide, pure 4-Benzylidene-2-methyl-oxazoline-5-one was observed as a yellow crystal (3.40 g, 53 %). mp: 139-141 °C.

Bacterial strains and culture

In this study, 10 reference microbial strains (9 bacterial strains and one yeast) were used including *Escherichia coli* strains ATCC 25922 and PTCC 1330, *S.aureus* strains ATCC 25923 and PTCC 1112, *Micrococcus luteus* PTCC 1110, *Bacillus cereus* PTCC 1015, *B.subtilis* PTCC1023, *Serratia marcescens* PTCC 1621, *Pseudomonas aeroginosa* ATCC 27853 and *Candida albicans* PTCC 5011. Moreover, five bacterial samples that were isolated from clinical samples were obtained from the microbiology laboratory of Afzalipour Hospital in Kerman, including *Proteus sp*, *P. aeroginosa*, *Enterococcus faecalis*, *Listeria monocytogenes*, *S. aureus*. The Mueller Hinton Agar/Broth medium (MHA/B; Merck) was used to culture for all bacteria except *E. faecalis* and *L. monocytogenes*, for which Brain Heart Infusion Agar/Broth medium (BHIA/B; Merck) was prepared. Also, Yeast Extract Glucose Chloramphenicol Agar medium (YGCA; Merck) and Potato dextrose broth (HiMedia) were applied to the yeast strain.

Antimicrobial activity assay

The antimicrobial activity was evaluated via the agar well diffusion method. To achieve this, a McFarland standard with a turbidity of 0.5 was prepared using an overnight culture of each microbial strain (18-24 hours) (Otter *et al*., 2013). About 1×10^6 CFU/ml microorganisms were incubated on the surface of the agar media by sterile swabs. The wells of 6 mm in diameter were created by a sterilized cork borer. In order to reveal its antimicrobial effect, 50 µL of the compound (50 mg dissolved in 1 mL DMSO) was added to the well. Then plates were incubated at 37 °C for 24- 48 h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone (IZ) that was formed around the well at the end of the incubation period. DMSO was used as a solvent and negative control and the penicillin G disk (10 units) and trimethoprim-sulfamethoxazole (SXT) disk (1.25+23.75 µg) were used as a standard antibiotic. Furthermore, fluconazole disk (25 µg) was used as a standard antifungal drug and as a positive control. The experiments were conducted threefold.

Determination of MIC and MBC

To determine the minimum efficient concentration of the compound to inhibit the microorganisms (MIC) and the minimum concentration of the ligand required to kill the microorganisms (MBC), the micro broth dilutions method was employed based on the National Committee for Clinical Laboratory Standards (CLSI) recommendations (CLSI, 2018). Mueller Hinton broth and potato dextrose broth were used for pathogen bacteria and *Candida albicans*, respectively (Kowalska-Krochmal and Dudek-Wicher, 2021; Parvekar *et al.*, 2020).

Monitoring biofilm formation

Afterward, we investigated the impact of BMO compound on bacterial biofilm formation using static 96-well microtiter plates (Kirmusaoğlu, 2019). We ascribed A595> 0.1 to the formation of biofilms since the values of negative control were shown less than 0.1.

Studybof the *icaA* gene expression of *S. aureus*

To study *icaA* gene expression in *S. aureus*, the following bacteria were used: two standard strains of *S. aureus* strains ATCC 25923 and PTCC 1112, and one strain of methicillin-resistant *S. aureus* isolated from the clinical sample. Bacteria were cultured in the MHB medium both in the presence (0.05 mg/mL) and absence (control) of the compound until reaching about OD_{600}= 0.4 turbidity. After that, the cells were collected via centrifuge (at 5000 g for 2 min). In due course, the plates were washed

twice with an ice-cold TE buffer. Total RNA was extracted via the extraction kit RNeasy Protect Mini kit (Qiagen) in accordance with the manufacturer's guidelines. Also, the reaction was treated with Deoxyribonuclease I (DNase I, RNase-free, Fermanias) according to manufacturers' instructions.

To synthesize cDNA from total RNA, 150 ng of total RNA and 0.50 mg primer OligodT were mixed at 42°C for 60 min using kit First Strand cDNA synthesis (Fermentas) according to the pre-determined instructions. The specific primers used for *icaA* in *staphylococcus* bacteria were consisted of forward (5'-AAACTTGGTGCGGTTACAGG-3') and Revers (5'' TCTGGGCTTGACCATGTTG-3'). The 16S rRNA gene was employed as the internal control gene. The primer of this gene was selected from Trotha/2001.

The qRT-PCR reaction was conducted using 7.50 µL of FastStart SYBR Green Master ROX (manufactured by Roche, Germany), 1.50 µL of cDNA, and 150 nM of each primer, resulting in a final reaction volume of 15 µL. The Rotor Gene 3000 instrument (manufactured by Corbett Research, Australia) was utilized for real-time PCR. The following conditions were applied: an initial denaturation step at 95 °C for 4 min, followed by 50 cycles of denaturation at 95 °C for 25 s, annealing at 54°C (temperature may vary based on the primers used) for 25 s, and extension at 72 °C for 30 s. Melting analysis was performed by heating the samples from 72 °C to 99 °C in 1-degree increments every 5 s. Data were collected during each extension step and melting analysis using the FAM/SYBR and ROX channels. The raw data obtained from the FAM/SYBR channel were normalized against the ROX channel to eliminate background fluorescence fluctuations. Subsequently, the data were exported to an Excel worksheet and analyzed using LinRegPCR software (version 11.1). The initial concentration of each sample was normalized against the corresponding internal control concentration to determine the actual expression value.

Cellular viability analysis

Cellular viability was assessed by MTT assay in which 2-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) reduces to insoluble form formazan by NAD (P) H dependent oxidoreductase enzymes in live cells (Denizot and Lang, 1986). MTT was dissolved in phosphate-buffered saline (PBS) and then introduced to the culture at a final concentration of 0.5 mg/mL. Following a 2-hour incubation period at 37 °C, the media were carefully aspirated, and 100 µL of dimethyl sulfoxide (DMSO) was added to the well. The absorbance values (OD) were measured at 490 nm using an automatic microplate reader (FLX 8000, Biotek). All experiments were performed six independent times, and 6 wells were selected for each group. The results were expressed as percentages relative to the control sample.

Statistical Analysis

All experiments underwent three repetitions. The data collected were analyzed using both SPSS Software (version 22) and Excel 2007. Statistical significance was determined for p-values below 0.05.

Results

Spectroscopic analysis of 4-Benzylidene-2-methyl-oxazoline-5-one

The structure of 4-benzylidene-2-methyl-oxazoline-5-one (BMO) was precisely characterized by ^{1}H NMR, ^{13}C NMR, and FT-IR. The ^{1}H NMR spectrum of this compound exhibited one singlet at $\delta = 2.73$ for the methyl group and one singlet at $\delta = 5.32$ for the methine group (Fig. 2). The ^{1}H-decoupled ^{13}C NMR spectrum of this compound exhibited 9 distinct resonances in agreement with the suggested structure. The characteristic ^{13}C NMR signals were displayed owing to two signals at over 160 ppm, being near each other. These chemical shifts can be assigned to the carbonyl group and the C-2 atom (Fig. 3).

Antimicrobial activity of BMO

As results indicate, the compound BMO has antibacterial effects on microorganisms (Fig. 4). Whereas only the two *B. cereus* PTCC 1015 and *B. subtilis* PTCC 1023 bacteria were sensitive to penicillin, 92.86 % of bacteria were resistant to this antibiotic (Table 1). According to CLSI in trimethoprim-sulfametoxazol (SXT), the inhibition zones ≤10 mm, 11-15 mm, and ≥16mm were reported to be resistant, semi-

sensitive, and sensitive, respectively. Therefore, 78.60% of the bacteria were identified to be sensitive to SXT. The results showed that this compound created a 12-15 mm inhibition zone in 78.60% of bacteria, and the diameter of the inhibition zone in 21.40 % of bacteria was ≥16 mm. The maximum inhibition zone created by this compound was attributed to *S. aureus* PTCC 1112 (IZ= 34 mm).

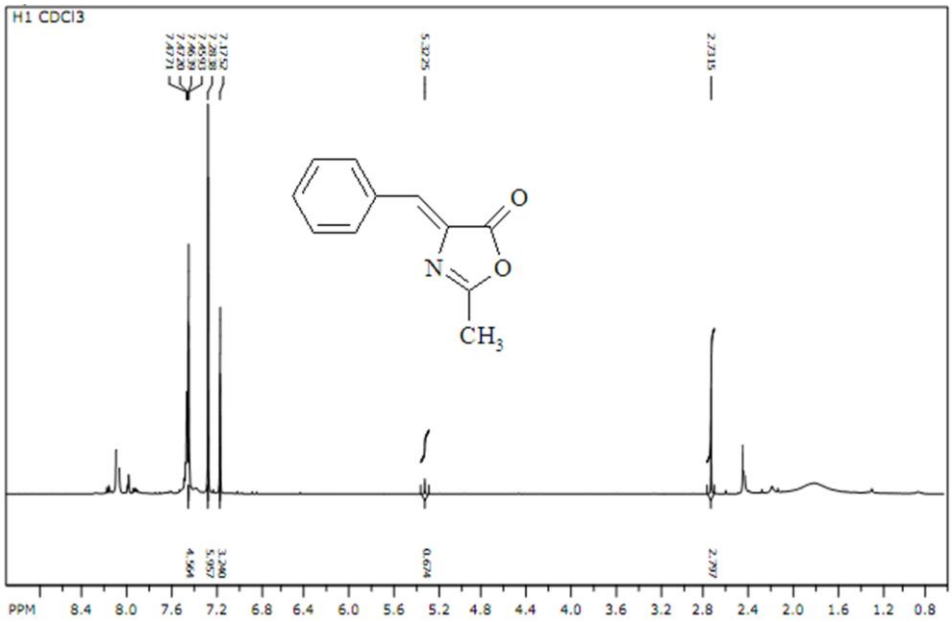

Fig. 2. [1]HNMR spectra for 4-benzylidene-2-methyl-oxazolin-5-one

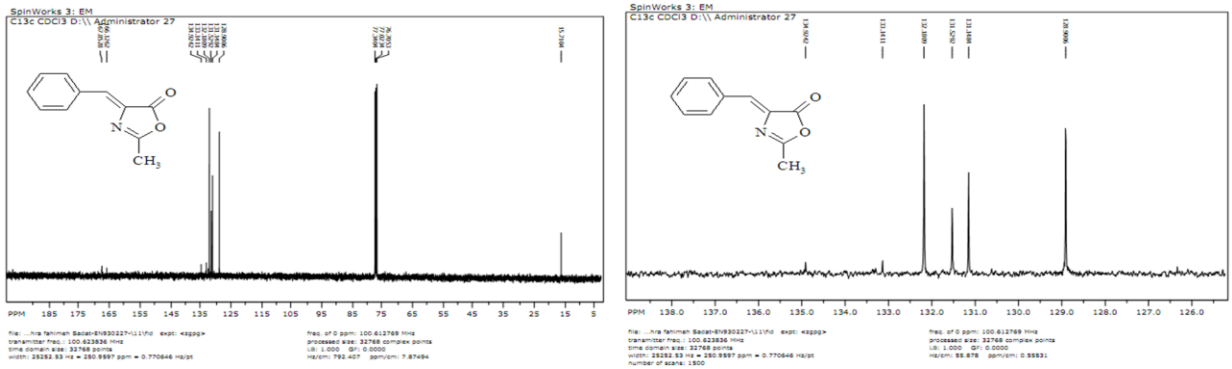

Fig. 3. [13]CNMR spectra for 4-benzylidene-2-methyl-oxazolin-5-one

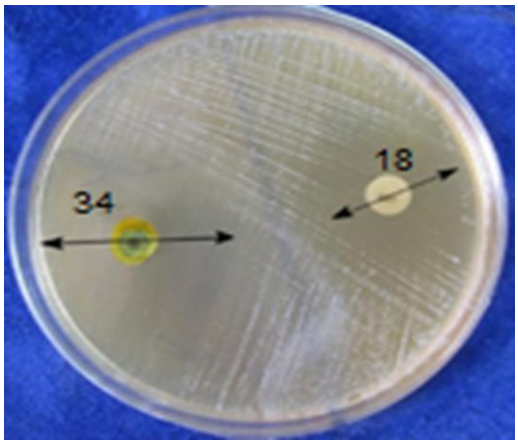

Fig. 4. Agar well diffusion method: Inhibition zone (IZ) for BMO in *S. aureus* PTCC 1112 (left) and SXT (right).

Table 1. *In Vitro* Antibacterial Activity of the Compound (4-benzylidene-2-methyl-oxazoline-5-one) (50 mg/ml), Inhibition Zone (IZ)

Microorganism	BMO	P[a]	SXT[b]	FL[c]	DMSO
E. coli ATCC 25922	12	0	25	0	0
P. aeroginosa ATCC 27853	12.5	0	23	0	0
S. aureus ATCC 25923	13	0	26	0	0
B. cereus PTCC 1015	21	15	20	0	0
B. subtilis PTCC 1023	16	16	22	0	0
E. coli PTCC 1330	13	0	22.5	0	0
M. luteus PTCC 1110	12	0	30	0	0
S. marcescens PTCC 1621	14	0	25	0	0
S. aureus PTCC 1112	34	0	18	0	0
*S. aureus**	15	0	18	0	0
*Proteus sp**	13	0	22	0	0
*Pseudomonas sp**	13	0	0	0	0
*E. faecalis**	13	0	0	0	0
*L. monocytogenes**	12	0	19	0	0
C. albicans PTCC 5011	14	0	0	32	0

*Isolated from the clinical sample; [a]penicillin G 10 units; [b]trimethoprime-sulfametoxazole (1.25+23.75 µg); [c]fluconazole (25 µg)

Interestingly, the two bacterial strains *P. aeroginosa* and *Proteus sp* which were isolated from clinical samples were resistant to SXT. However, they were highly sensitive to 4-benzylidene-2-methyl-oxazoline-5-one (Table 2). Also, BMO had an antimicrobial effect on the yeast *C. albicans* PTCC 5011, as a eukaryotic cell.

Based on the MIC/MBC testing results, it was found that BMO has both bacteriostatic and bactericidal effects on 86.67% of the tested microorganisms. However, it only exhibits a bactericidal effect on 13.32% of the microorganisms (Table 2). Additionally, the results indicate that BMO effectively prevents biofilm formation in *S. aureus* ATCC 25923 (0.05 mg/ml), *M. luteus* PTCC 1110 (0.05 mg/ml), clinical *S. aureus* (0.05 mg/ml), and *Proteus sp*. (1.6 mg/ml) at concentrations below the MIC (sub-MIC) (Table 2).

Table 2. *In Vitro* Antibacterial Activity of compound 4-benzylidene-2-methyl-oxazoline-5-one (MIC, MBC, and BF: mg/ml)

Microorganism	BMO		
	MIC*	MBC**	BF***
E. coli ATCC 25922	0.4	12.5	_
P. aeroginosa ATCC 27853	0.2	12.5	_
S. aureus ATCC 25923	6.25	13.12	0.05
B. cereus PTCC 1015	_	0.05	_
B. subtilis PTCC 1023	_	0.05	_
E. coli PTCC 1330	0.4	12.5	_
M. luteus PTCC 1110	0.1	6.25	0.05
S. marcenscens PTCC 1621	0.8	6.25	_
S. aureus PTCC 1112	0.05	0.2	_
*S. aureus**	0.4	6.25	0.05
Proteus sp	3.12	6.25	1.6
*Pseudomonas sp**	3.12	6.25	_
*E. faecalis**	0.05	3.12	_
*L. monocytogenes**	0.05	0.1	_
C. albicans PTCC 5011	0.05	6.25	_

*MIC: Minimum Inhibitor Concentration; **MBC: Minimum Bactericidal Concentration; ***BF: Biofilm Formation

The *icaA* gene expression of *S. aureus*

The morphological studies on BMO revealed that BMO has prevented biofilm formation in *S. aureus* when compared to the control group (Fig. 5A). According to the results of real-time PCR, it was found that BMO effectively suppresses the expression of the *icaA* gene (Fig. 5B). The influence of BMO on the expression of the *icaA* gene was observed in all three strains, namely *S. aureus* strains ATCC 25923 and PTCC 1112, as well as *S. aureus* strains isolated from clinical samples. The data revealed that the presence of BMO led to a decrease in the expression of the *icaA* gene compared to the control group without BMO (Fig. 5B). Statistical analysis also demonstrated a significant difference between the stress condition (treatment with BMO) and the control group (without BMO) ($p < 0.05$).

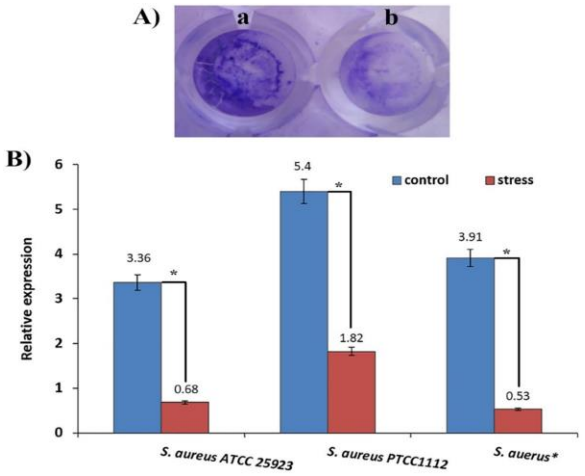

Fig. 5. Effect of the BMO: A) Phenotypical study of biofilm formation in *S. aureus* ATCC 25923. (a) Control (without the BMO). (b) Stress (In the presence of BMO); B) Measurement of *icaA* gene expression. The stress (with BMO) and control (without BMO). Error bars represent standard deviations of the mean values of results from three independent experiments. This difference was statistically significant ($p< 0.05$).

Toxicity analysis

At concentrations of 0.01, 0.1, and 1 mg/mL, 4-benzylidene-2-methyl-oxazoline-5-one induced cell toxicity and decreased cellular viability. However, no significant effects on MCF-7 cell viability were observed at a concentration of 0.05 mg/mL (Fig. 6). The results suggest that MIC concentrations do not have a cytotoxic effect, while cellular death occurs at higher concentrations.

Discussion

The breakthrough discovery of antibiotics, beginning with penicillin in 1928, brought about a revolutionary transformation in the management of bacterial infections, leading to the preservation of countless lives (Crofts *et al.*, 2017). However, the overuse and improper use of antibiotics have led to the emergence of bacterial resistance. The formation of bacterial biofilms is one of the main causes of bacterial resistance (Bridier *et al.*, 2011). The search for new antimicrobial compounds to combat antibiotic resistance is a global priority. Oxazoline compounds have gained attention for their ability to inhibit biofilm formation and disrupt established biofilms.

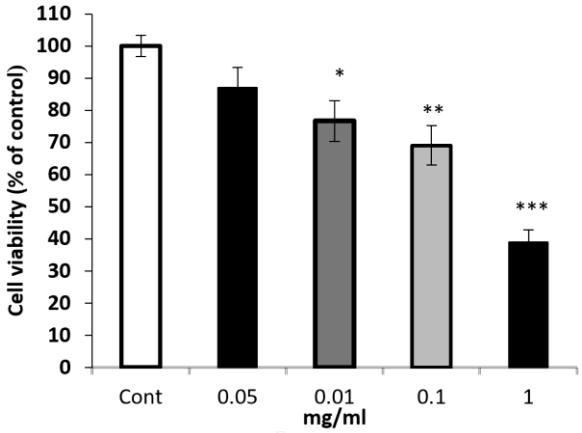

Fig. 6. The effects of 4-benzylidene-2-methyl-oxazoline-5-one on the viability of breast cancer MCF-7 cell line were determined by MTT assay. Data are expressed as mean ± SEM; n = 5-6 wells for each group. *P<0.05, **P<0.01 ***P<0.001 versus control cells.

Studies have shown that these compounds possess significant antimicrobial activity against a wide range of pathogenic bacteria, including both Gram-positive and Gram-negative strains. These compounds can inhibit bacterial adhesion to surfaces, disrupt the extracellular matrix, and inhibit the formation of biofilm-associated structures such as quorum-sensing molecules and exopolysaccharides (Bala *et al*., 2011). In this work 4-Benzylidene-2-methyl-oxazoline-5-one,

a molecule containing oxazoline, exhibits notable antibacterial and anti-biofilm properties. it is noteworthy that the two bacterial strains P. *aeruginosa* and *Proteus sp*, which were obtained from clinical samples, displayed resistance to SXT. Conversely, they exhibited a remarkable sensitivity to 4-benzylidene-2-methyl-oxazoline-5-one. Moreover, this compound demonstrated antimicrobial properties against the eukaryotic cell *C. albicans* PTCC 5011, a yeast species. Also, this compound shows bacteriostatic and bactericidal effects in most microorganisms and also prevents biofilm formation in *Staphylococcus aureus* ATCC 25923, *M. luteus PTCC* 1110, clinical *Staphylococcus aureus* and *Proteus sp* at concentrations lower than MIC. A study conducted by Pasha et al. examined the *in vitro* antibacterial activity of 4-arylmethylidene-2-phenyl 5(4H)-oxazolones against *B. subtilis* and *E. coli*. The findings of this study demonstrated the antibacterial activity of the compound against both bacterial strains (Pasha *et al*., 2007). Also, Fawzi et al. developed novel isoxazoline-1,3,4-thiadiazole hybrids from (S)-verbanone for potential anticancer therapy, particularly focusing on cytotoxic and apoptotic effects in hormone-sensitive MCF-7 and triple-negative MDA-MB-231 (Fawzi *et al*., 2023). These findings showed that (S)-verbenone isoxazoline-1,3,4-thiadiazole derivatives have good potential for breast cancer treatment due to their remarkable apoptotic activity. In another experiment that was conducted to investigate the antimicrobial and anti-biofilm effect of 4H-1,3-oxazol-5-ones and its derivatives against Gram-positive, Gram-negative, and fungal strains, the results showed a good antimicrobial effect for such compounds (Apostol *et al.*, 2022).

Our findings demonstrated that the expression of the *icaA* gene in *S. aureus* exerts a significant influence on biofilm formation. Additionally, compelling evidence indicates that external stresses can influence both biofilm formation and the expression of the *ica* gene. In line with these observations, Rachid et al. reported that sub-inhibitory concentrations of tetracycline and the semisynthetic streptogramin antibiotic quinupristin-dalfopristin significantly enhanced the expression of the *ica* gene. Conversely, penicillin, oxacillin, chloramphenicol, gentamicin, ofloxacin, and vancomycin had no discernible impact on *ica* expression (Rachid *et al*., 2000). Additionally, it has been shown that alcohol treatment can simultaneously enhance biofilm formation and the expression of the *ica* gene in *S. aureus*. (Redelman *et al*., 2012).

4-benzylidene-2-methyl-oxazoline-5-one offers the advantage of exhibiting equal efficacy against both Gram-positive and Gram-negative bacteria. In contrast to antibiotics like penicillin, which rely on a β-lactam ring to target the cell wall, this compound demonstrates notable effectiveness against Gram-negative bacteria in addition to its impact on Gram-positive bacteria. This broad-spectrum activity makes it a valuable option for addressing infections caused. Cycloserine, a versatile antibiotic, exhibits both bactericidal and bacteriostatic properties. It functions as an analog of D-alanine, an amino acid, and disrupts an initial stage of bacterial cell wall synthesis within the cytoplasm. This disruption occurs through competitive inhibition of two enzymes: 1. L-alanine racemase, responsible for converting L-alanine to D-alanine, and 2. D-alanine-D-alanine synthetase, which incorporates D-alanine into the pentapeptide is necessary for the formation of peptidoglycan and subsequently, synthesis of the bacterial cell wall (Prosser and de Carvalho, 2013).

Given the structural similarity between 4-benzylidene-2-methyl-oxazoline-5-one and the antibiotic cycloserine, it is reasonable to propose that this compound may exert similar effects and employ comparable mechanisms as cycloserine. It is known to disrupt bacterial cell wall synthesis in the cytoplasm through competitive inhibition of the enzymes L-alanine and racemase. Therefore, it is plausible that 4-benzylidene-2-methyl-oxazoline-5-one operates in a similar manner. However, the underlying mechanism by which the given compound exerts its inhibitory effects on microorganisms remains largely unknown. Further studies are needed to expand our understanding of the therapeutic potential of 4-benzylidene-2-methyl-oxazoline-5-one and its potential application in clinical phases.

Conclusion

By study thorough investigation into the antimicrobial properties of a specific oxazoline

derivative, 4-benzylidene-2-methyl-oxazoline-5-one, against ten reference microbial strains and 5 bacterial samples isolated from clinical sources, noteworthy antimicrobial activity was observed. Particularly, this compound exhibited significant efficacy against Gram-positive bacteria. Additionally, it displayed inhibitory effects on biofilm formation in 40% of the microorganisms at sub-MIC concentrations, highlighting its potential as an agent to impede biofilm development. Furthermore, based on the real-time PCR results, an interesting finding emerged regarding the relationship between the expression of the *icaA* gene and the concentration of 4-benzylidene-2-methyl-oxazoline-5-one in *S. aureus*. It has been observed that the presence of BMO resulted in a significant decrease in the expression level of the *icaA* gene, in comparison to the control group where BMO was absent.

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Disclosure Statement

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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