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Evaluation of Antibacterial and Anticandidal Activities of Some Imidazole, Benzimidazole and Benztriazole Derivatives

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Abstract

In recent years, resistance to conventional antimicrobial drugs has become a serious concern in the clinic. Hence, the discovery of novel and effective antimicrobial agents with improved properties is of great value. In this study, the antibacterial and anticandidal activities of some imidazole, benzimidazole and benztriazole derivatives (C1-C8) against several species of Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Salmonella typhi* as well as one species of fungi including *Candida albicans*, were evaluated using Clinical & Laboratory Standards Institute (CLSI) method. Antimicrobial evaluation revealed that among the tested compounds, imidazole derivative (C5) indicated the highest activity against bacterial strains and *Candida albicans*. It was found that compound C5 is more potent than ampicillin against *S. aureus* strain (MIC =2-16 µg/mL) and possessed excellent anticandidal activity (MIC =1 µg/mL) which was comparable to Amphotericin B as the positive control. In addition, cytotoxic activity of the tested compounds was investigated using MTT assay. The results of MTT assay demonstrated that the toxicity of the tested compounds was less than 35%.

Keywords: Azoles, Antimicrobial Effect, Antifungal Activity, MTT

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1. Introduction

In recent decades, infections caused by multidrug-resistant bacteria have been a major public health concern which has been associated with high rates of mortality in both humans and animals (1, 2). Fungal infections, especially those caused by Candida species, have also been considered an important health challenge worldwide (3, 4). It was reported that candida cause more than 350,000 deaths annually, and this number is expected to increase significantly because the available therapy is not effective enough in the treatment of infection caused by multidrug resistance species (5). Hence, the discovery of novel and effective antimicrobial agents with improved properties such as high efficacy, broad-spectrum, low toxicity and low resistance is of great value (6).

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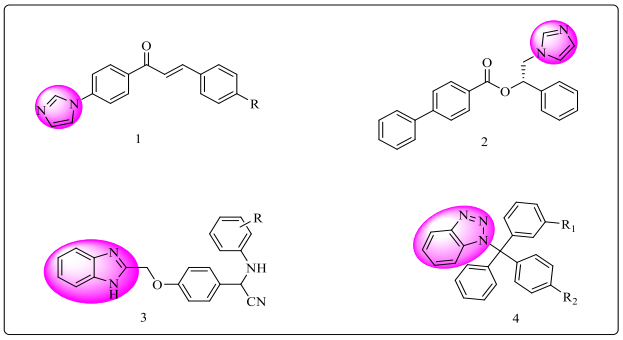


Figure 1. Structures of azole derivatives as antibacterial and antifungal agents.

Among pharmaceutical compounds that are used as antimicrobial agents, azole derivatives such as imidazole and triazole derivatives have received great attention for the synthesis of more effective antifungal and antibacterial agents with extensive spectrum (7, 8). Currently, a great number of azole agents are used clinically for the treatment of fungal infections. Miconazole, econazole, clotrimazole, ketoconazole, itraconazole and fluconazole are examples of azoles use as antifungal agents. The antibacterial activity of azoles has also been reported. For example, miconazole has exhibited remarkable potential against methicillin-resistant *S. aureus* (MRSA) (6).

The antifungal activity of azoles is due to their potential to bind and inhibit cytochrome P450 14α-demethylase (CYP51) enzyme. Inhibition of CYP51 enzyme prevents the conversion of lanosterol to ergosterol. Ergosterol is an essential component of the fungal cell membrane which inhibition of its biosynthesis leads to the fungal cell death. Furthermore, azoles exert their antibacterial activity by inhibiting a protein called acyl reductase transporter (FabI), which is involved in the synthesis of fatty acids (7-10).

In recent years, a wide range of azole derivatives have been reported to exhibit various biological activities such as antifungal and antibacterial activities which make them an important scaffold for design and synthesis of potential antimicrobial agents (Figure 1) (8, 10-15).

In this study, the antibacterial and anticandidal activities of some previously synthesized azole derivatives (16), were investigated. These azole derivatives contain imidazole, benzimidazole and benztriazole moieties. The target compounds were tested against four bacterial species and one fungal strain using the broth microdilution method as recommended by Clinical & Laboratory Standards Institute (CLSI). Furthermore, these compounds were evaluated for their cytotoxic activities against HepG2 cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. In order to determine the rates at which bacteria are killed after exposure to the tested compounds, time-kill studies were performed

2. Material and methods

All reagents and solvents were obtained from Merck Company. Novel synthesized compounds C1-C8 were obtained from Department of Medicinal Chemistry, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran (16).

2.1. Microorganisms

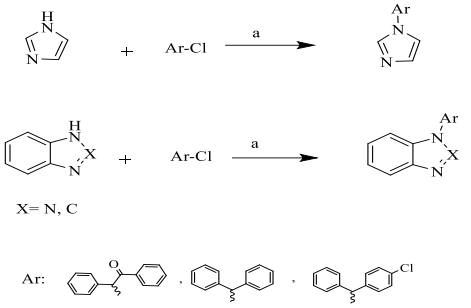
Four bacteria strains comprised of both Gram-positive and Gram-negative including *Staphylococcus aureus* (ATCC 1112), *Escherichia coli* (ATCC1329), *Salmonella typhi* (ATCC 1609) and *Streptococcus pyogenes* (ATCC 1447), as well as one fungal species, *Candida albicans* (ATCC 5027), were tested.

2.2. Cell line

Hep G2 cell line was purchased from the National Cell Bank of Pasteur Institute of Iran (Tehran, Iran).

2.3. Chemistry

Compounds C1-C8 were synthesized according to the method that described previously (16). Briefly, a mixture of imidazole or triazole analogs, aryl halide, potassium carbonate (K_2CO_3),



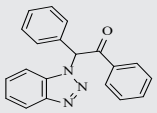
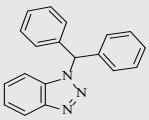
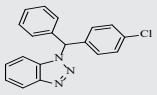
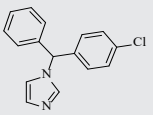
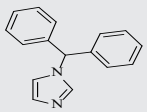
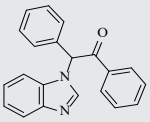
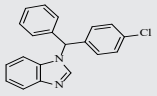
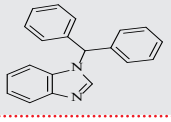
Scheme 1. Synthesis of azole derivatives. a: NaOH, K_2CO_3, TEAI, acetonitrile.

tetraethylammonium iodide (TEAI), and sodium hydroxide (NaOH) were dissolved in acetonitrile solvent and refluxed at 85 °C for 24-48 h. Upon completion of the reaction, the reaction mixture was filtered and the resulting solid was extracted with dichloromethane. Subsequently, the extracted organic layer was purified by column chromatography using chloroform/ethanol (9.3:0.7) as eluent for imidazole derivatives and dichloromethane/ethyl acetate (9:1) as eluent for benzimidazole and benzotriazole derivatives to afford the desired compounds C1-C8 (Scheme 1). The structure of compounds C1-C8 is shown in Table 1.

2.4. Antimicrobial activity

The *in vitro* antibacterial and anticandi-

Table 1. The structures and chemical properties of tested compounds.

Entry	Chemical Structure	Chemical Name	M.W.	M.P. (˚C)	Log P
C1		2-(1H-benzo[d][1,2,3]triazol-1-yl)-1,2-diphenylethan-1-one	313.36	142-145	4.26
C2		1-benzhydryl-1H-benzo[d][1,2,3] triazole	285.35	116-118	4.90
C3		1-((4-chlorophenyl) (phenyl)methyl)-1H-benzo[d][1,2,3]triazole	319.79	115-118	5.46
C4		1-((4-chlorophenyl) (phenyl) methyl)-1H-imidazole	268.74	110-112	3.57
C5		1-benzhydryl-1H-imidazole	234.30	80-83	3.02
C6		2-(1H-benzo[d]imidazol-1-yl)-1,2-diphenylethan-1-one	312.37	148-150	3.85
C7		1-((4-chlorophenyl) (phenyl)methyl)-1H-benzo[d]imidazole	318.80	110-113	5.05
C8		1-benzhydryl-1H-benzo[d]imidazole	284.36	147-150	4.49

dal potential of the synthesized compounds (C1-C8) were evaluated using the broth microdilution method (CLSI) with brief modifications (17, 18). To determine the Minimum Inhibitory Concentrations (MICs) of the target compounds against the tested bacteria and fungi strains, serial dilutions of the tested compounds (1-1024 µg/mL) were prepared in 96-well microtiter plates, using Roswell Park Memorial Institute (RPMI- 1640) media buffered with 3-(N-morpholino) propanesulfonic acid (MOPS) for bacteria and in the Muller-Hinton media for fungi. In the following, stock inoculums of bacteria and fungi were prepared by suspending three colonies of the tested microorganisms in 5 mL sterile 0.85 % NaCl and adjusted to the turbidity of 0.5 McFarland standard at 530 nm wavelength. The final concentration of bacteria and fungi were 1-5×10^6 cells/mL and 1-1.5×10^8 cells/ml, respectively. To prepare the working suspension, the stock suspension was 1000 times diluted with either RPMI or Muller-Hinton broth for fungi and bacteria, respectively. The conidia were collected and then transferred to sterile saline solution. The turbidity was adjusted to 0.09-0.11 optical density to obtain 0.4-5×10^6 conidia/mL. 100 µL of the working suspension was added to each well of microtiter plate. The plates were then incubated for 24-48 h at 30 °C for fungi, or for 24 h at 37 °C for bacteria, in a humid atmosphere. Uninoculated medium and medium with inoculums but without the compounds were also included as sterility (blank) and growth controls respectively. MICs were defined as the lowest concentration of the tested compounds that inhibit the growth of microorganism. Each experiment was carried out in triplicate and means were calculated.

2.5. Time-kill assay

The time-kill assay was performed following the method that was previously reported (19). Briefly, 3-5 colonies of *E.coli* and *S.aureus* species isolates grown on sabouraud dextrose agar (SDA) for 24 to 48 h. Then, the bacterial concentration was counted using a hemocytometer. Dilutions resulted in a starting inoculum of approximately 1×10^6 CFU/mL. *S. aureus* and *E. coli* were exposed over time to compounds C5 and C7. Test samples were placed on a shaker and incubated for 0, 0.5, 1, 4, 12, 24 and 48 h at 37 °C. After that, an amount of 100 µL was removed from each test suspension, serially diluted with sterile saline and plated on potato dextrose agar (PDA) for colony counting. Plates were incubated for 24 h at 37 °C. The experiments were performed in triplicate.

2.6. Cytotoxicity evaluation

The potential cytotoxicity of the synthesized derivatives (C1-C8) was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. In brief, HepG2 cells were seeded in 96-well plates containing RPMI 1640, with a density of 1.0×10^4 cells/well and incubated for 24 h at 37 °C and 5% CO_2. After this time, the medium was removed and replaced with the fresh medium containing different concentrations of compounds and incubated for 24 h. Following the treatment, the cells were incubated with MTT (0.5 mg/ ml) at 37 °C for 4 h. The medium was then removed and 100 µL of DMSO were added into each well. The absorbance of wells was measured at the wavelength of 540 nm using a microplate spectrophotometer. The cell viability (%) was calculated according to the equation 1 (20).

$$Cell\ viability = (Test\ OD)/(Control\ OD)\times 100 \quad \text{(Eq. 1)}$$

3. Results and discussion

3.1. Antibacterial activity

The *in vitro* antibacterial activities of the tested compounds (C1-C8) against two Gram-positive bacteria (*S. aureus* and *S. pyrogen*) and two Gram-negative bacteria (*E. coli* and *S. typhi*) were investigated using CLSI method. In this assay ampicillin used as positive control. MIC values of the tested compounds against four bacteria strains are summarized in table 2. According to the biological results, among these derivatives, C5, C6 and C7 showed the highest antibacterial activities. Compound C5 indicated the best inhibitory activity against Gram-positive bacteria with MIC values of 1-4 µg/mL and against Gram-negative bacteria with MIC values of 8-64 µg/mL. Compound C5 was found to be more potent than Ampicillin against *S. aureus* strain. Compound C6 exhibited the MIC values of 32-128 µg/mL against Gram-

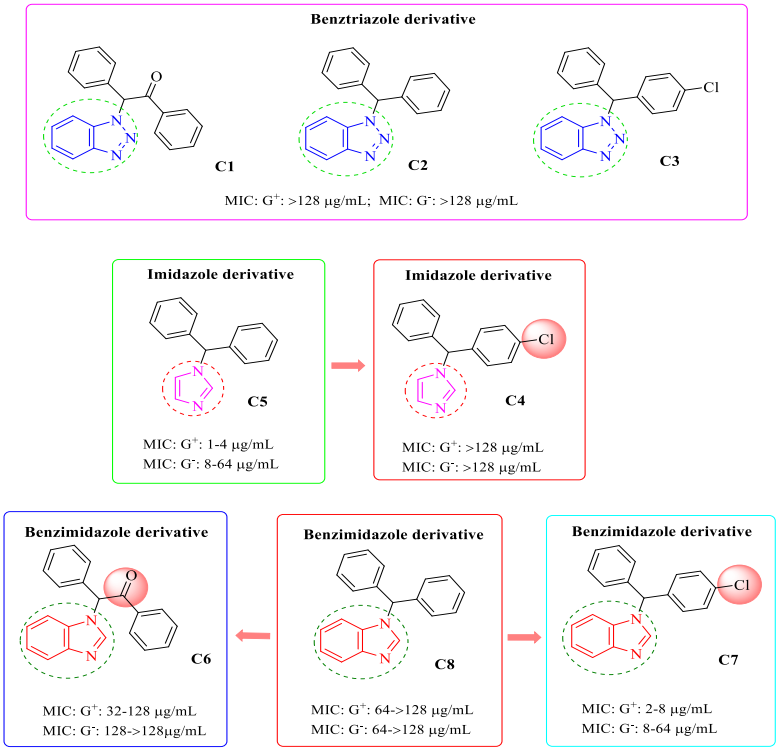


Figure 2. Antibacterial activities of the tested compounds C1-C8 against Gram-positive (G+) and Gram-negative (G-) bacteria species including *S. aureus*, *S. pyrogen*, *E. coli* and *S. typhi*. MIC values of the tested compounds were determined using CLSI method. Each experiment was carried out in triplicate and means were calculated.

positive bacteria and the MIC values of 128 µg/mL against Gram-negative bacteria. Compound C7 displayed the MIC values of 2-8 µg/mL against Gram-positive bacteria and MIC values of 8-64 µg/mL against Gram-negative bacteria. Compound C8 inhibited the growth of tested bacteria strains at MIC values of 64-128 µg/mL. Other compounds showed no antibacterial activities (Table 2).

Compound C5 with imidazole moiety, likely due to its small size and subsequently better penetration into the bacterial cell (21, 22), showed the best antibacterial activity. It was observed that in the case of imidazole derivatives, the presence of chlorine substituent at the para position of the phenyl ring eliminated the antibacterial activity (compound C5 vs. C4). Furthermore, it was observed that the presence of a ketone group in the structure of compound C6 improved antibacterial activity. The ketone group creates a better balance between hydrophilicity and lipophilicity and subsequently improved antibacterial activity. Compound C7 is a benzimidazole derivative with a chlorine substituent at para position of phenyl ring. It was observed that in the case of benzimidazole derivatives, the presence of chlorine substituent on the phenyl ring due to increased polarity, improved antibacterial activity and the elimination of chlorine substituent from the phenyl ring significantly reduced antibacterial activity (compound C7 vs. C8) (Figure 2).

The antibacterial activities demonstrated that the C5 derivative was more active against the tested bacteria, especially gram positive bacteria,

Table 2. The MIC values (µg/mL) of the compounds C1-C8 against tested bacteria strains.

Compounds		C1	C2	C3	C4	C5	C6	C7	C8	Ampicillin
Bacteria										
G^+	*S. aureus*	>128	>128	>128	>128	1-2	64-128	2-4	>128	16-32
	S. pyrogen	>128	>128	>128	>128	2-4	32-64	4-8	64-128	2-4
G^-	*E. coli*	>128	>128	>128	>128	32-64	128	8-16	64-128	8-16
	S. typhi	>128	>128	>128	>128	8-16	>128	32-64	>128	4-8

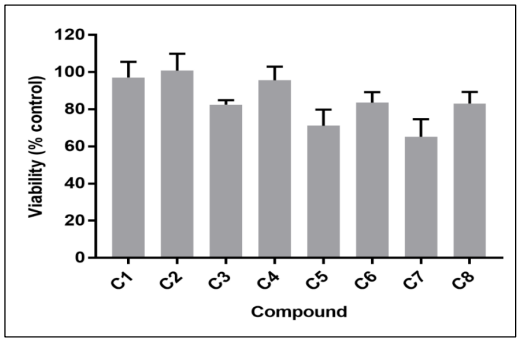


Figure 3. Cytotoxic activity of the tested compounds C1-C8 against HepG2 cell evaluated using MTT assay at 250 µg/mL concentration for 24 h. Data are expressed as means ±SD. from three separate experiments.

compared to the similar synthesized compounds which previously reported (10, 15, 23-25). This finding suggested that the imidazole ring is more favorable for the antibacterial activity and the small size of molecules exerted a positive effect on the enhancement of the antibacterial activity.

3.2. Antifungal activity

The *in vitro* antifungal activities of the tested compounds (C1-C8) against *Candida albicans* (*C. albicans*) were investigated. The MIC values of the tested compounds and Amphotericin B as standard drug are summarized in table 3. According to the results, all compounds except compounds C1 and C4 exhibited antifungal activity against C. albicans. Among active compounds, compound C5 with MIC value of 1 µg/mL displayed excellent antifungal activity. The MIC value of compound C5 was comparable to that of Amphotericin B as controlled drug. Compound C5 with imidazole moiety, due to its small size, has a better penetration into the fungi cell. Furthermore, compound C7 as a benzimidazole derivative showed MIC value of 4-8 µg/mL against C. albicans. This result demonstrates that in the case of benzimidazole derivatives, the presence of 4-Cl substituent on the phenyl ring improves antifungal activity. Other compounds (C2, C3, C6-C8) showed moderate activity when compared to Amphotericin B.

The results of anticandidal activity indicated that the synthesized compounds exhibited moderate to good activity against *C. albicans*. Among all the synthesized compounds, C5 derivative showed excellent anticandidal activity compared to the similar synthesized compounds (8, 12, 23, 26), which revealed the small size of molecules exerted a positive effect on the enhancement of activity.

3.3. Time-kill studies

The time-kill assay was carried out to determine the (bactericidal activities of tested compounds towards *S. aureus* and *E. coli* strains) rates at which bacteria were killed by exposure to the tested compounds. In the current study, the time-kill studies of compounds C5 and C7, which had shown the highest activities against bacteria strains, were performed against *S. aureus* and *E. coli* strains at 8, 32 and 128 µg/mL concentrations of these compounds. As demonstrated in table 4, the time-kill kinetics profile tested compounds showed a time and dose-dependent manner reduction in the number of viable cells. Approximately, for all concentrations (except for compound C5), the maximum reduction in the number of viable cells for both *E. coli* and *S. aureus* strains was observed at 24 h.

3.4. Cytotoxic activity

The main cause of failure in the discovery of new drugs is toxicity; therefore, the selectivity

Table 3. The MIC values (µg/mL) of the tested compounds C1-C8 against *C. albicans*.

Compounds	C1	C2	C3	C4	C5	C6	C7	C8	Amphotericin B
Fungi									
C. albicans	>128	64-128	32-64	>128	1	64-128	4-8	8-16	2-4

Table 4. Result of time-kill assay using compounds C5 and C7 against *E. coli* and *S. aureus*.

Bacteria	Compounds	Concentration (µg/mL)	Number of bacteria (CFU/mL) Time (h) 0	0.5	1	4	12	24	48
S.aureus	C5	8	$1\pm0.22\times10^8$	$3\pm0.65\times10^7$	$2\pm0.22\times10^3$	416±28	65±5	ND	ND
		32	$1\pm0.22\times10^8$	$1\pm0.95\times10^5$	$3\pm0.55\times10^3$	93 ±11	ND	ND	ND
		128	$1\pm0.22\times10^8$	$2\pm0.5\times10^4$	$1\pm0.05\times10^3$	193±11	ND	ND	ND
	C7	8	$1\pm0.22\times10^8$	$1\pm0.65\times10^7$	$5\pm.55\times10^3$	600±11	85±5.7	ND	ND
		32	$1\pm0.22\times10^8$	$2\pm0.55\times10^6$	$4\pm0.11\times10^3$	233±28	83±8.1	ND	ND
		128	$1\pm0.22\times10^8$	$5\pm0.45\times10^5$	$2\pm0.45\times10^3$	230±12	76±11	ND	ND
E.coli	C5	8	$1\pm0.09\times10^8$	$1\pm0.05\times10^5$	$3\pm0.55\times10^4$	684±11	83±8	ND	ND
		32	$1\pm0.09\times10^8$	$1\pm0.55\times10^5$	$2\pm.065\times10^4$	254±11	93±6	ND	ND
		128	$1\pm0.09\times10^8$	$2\pm.21\times10^5$	$1\pm0.69\times10^4$	165±28	103±6	ND	ND
	C7	8	$1\pm0.09\times10^8$	$5\pm0.58\times10^6$	$5\pm0.12\times10^4$	546±11	406±5	ND	ND
		32	$1\pm0.09\times10^8$	$4\pm0.56\times10^5$	$2\pm0.21\times10^3$	554±28	103±4.5	ND	ND
		128	$1\pm0.09\times10^8$	$1\pm0.46\times10^5$	$3\pm0.56\times10^3$	456±15	93±5.7	ND	ND

ND: Not Detected; Results are represented as mean±SEM.

of antimicrobial agents towards the microorganism while not affecting host cells, is very important (8, 27). In this study, the toxicity of compounds C1-C8 was investigated against HepG2 cells according to the MTT assay at 250 µg/mL concentration. The cytotoxicity result of the tested compounds is depicted in Figure 3. The MTT assay results indicated that in the case of compounds C5 and C7, the viability of HepG2 cells was 71% and 65%, respectively, and other compounds had maximum toxicity up to 20% at the same concentration. The obtained results suggested that these compounds exhibited low toxicity to the host cells at concentrations below 250 µg/mL. Furthermore, these observations demonstrate that the antimicrobial activities of the tested compounds C1-C8 is not due to general toxicity but can be attributed to their selective action towards bacterial and candida strains.

4. Conclusion

In conclusion, the antimicrobial activities of some azole derivatives against four species of bacteria and one species of fungi were evaluated. Among the tested compounds, compound C5 indicated the best inhibitory activity against tested bacteria strains. According to the obtained MIC values, C5 was more potent than Ampicillin against *S. aureus* strain. In addition, compound C5 possessed excellent activity against *C. albicans* which was comparable to Amphotericin B. The obtained results revealed that the imidazole fragment, because of its small size and subsequently better penetration into the bacteria and fungi cells is more favorable than benzimidazole and benztriazole scaffolds for antibacterial and antifungal activities and the presence of chlorine substituent on the phenyl ring in benzimidazole derivatives due to increased polarity, improved antibacterial and antifungal activities and elimination of chlorine substituent from the phenyl ring significantly reduced antibacterial activity. In addition, MTT assay was performed to investigate the cytotoxicity of the tested compounds. The MTT results showed that the toxicity of the tested compounds was less than 35%. According to the biological results, compound C5 can be considered as the most promising antimicrobial agent to develop new antimicrobial agents with higher potency in the future.

Acknowledgment

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Conflict of Interest

None declared

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