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DESIGN AND ANTIMICROBIAL EVALUATION OF 1-METHYLIMIDAZOLE DERIVATIVES AS NEW ANTIFUNGAL AND ANTIBACTERIAL AGENTS

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Azole antifungal agents, which are widely used as antifungal antibiotics, inhibit cytochrome P450 sterol 14α-demethylase (CYP51). In this research, a group of 1-methylimidazole derivatives were synthesized for evaluation as antibacterial and antifungal agents. Antimicrobial evaluation revealed that some of these compounds exhibited significant antimicrobial activities against tested pathogenic fungi and bacteria, wherein compounds **3** and **8** were most potent. To find the action mechanism, all of these compounds were subjected to docking studies using the AutoDock 4.2 program. The results show that all of the azoles (2 – 5, 7, and 8) interacted with 14α-DM, wherein azole – heme coordination, hydrogen binding, and π-cation interactions were involved in the drug – receptor interaction. These studies offer some useful references in order to understand the action mechanism; moreover, performing the molecular design or modification of this series as a lead compound can assist in identifying new and potent antimicrobial agents.

Keywords: antibacterial, antifungal, azole, docking, imidazole

INTRODUCTION

Research and development of potent and effective antimicrobial agents is an important step in advancing therapeutics. Over the past decade, fungal infections have become a major complication and cause of morbidity and mortality in immunocompromised individuals, such as those suffering from tuberculosis, cancer, acquired immune deficiency syndrome (AIDS), and in organ transplant cases [1]. In these hosts with impaired immune systems, fungal pathogens can easily invade into tissues and cause serious infections with higher rates of morbidity and mortality [2, 3]. Among these, *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* are the most common causes of invasive fungal infections [4, 5]. In clinics, antifungal agents that can be used to treat life-threatening fungal infections are limited. These drugs fall into five major classes: azoles, allylamines, polyenes, fluropyrimidines, and thiocarbamates [6]. Among these classes, azoles are the most widely used antifungal agents because of their high therapeutic index.

Azoles, imidazoles and triazoles (Fig. 1) constitute a large and relatively new group of synthetic compounds that are used in the treatment of systemic fungal infections as well as in agriculture [7 – 9]. Lanosterol-14α-demethylase (14α-DM, CYP51) is a key enzyme of sterol biosynthesis in fungi [10]. Azole antifungal agents inhibit 14α-DM by a mechanism in which the heterocyclic nitrogen atom (N-3 of imidazole and N-4 of triazole) coordinates to the heme in the active site of the enzyme. The resulting ergosterol depletion and the accumulating of precursor 14α-methylated sterols disrupt the structure of the plasma membrane, making it more vulnerable to further damage, and alter the activities of several membrane-bound enzymes [11, 12].

The efficacy of azoles depends on the strength of the binding to heme iron as well as the affinity of the N-1 substituent for the cytochrome protein [13]. Because of the existence of CYP51 in fungi and mammalians and the effects of these compounds on CYP3A4, the selective inhibition of 14α-DM in fungi is very important and results in an increased therapeutic index [13 – 16]. However, the extensive use of azoles has led to the development of severe resistance

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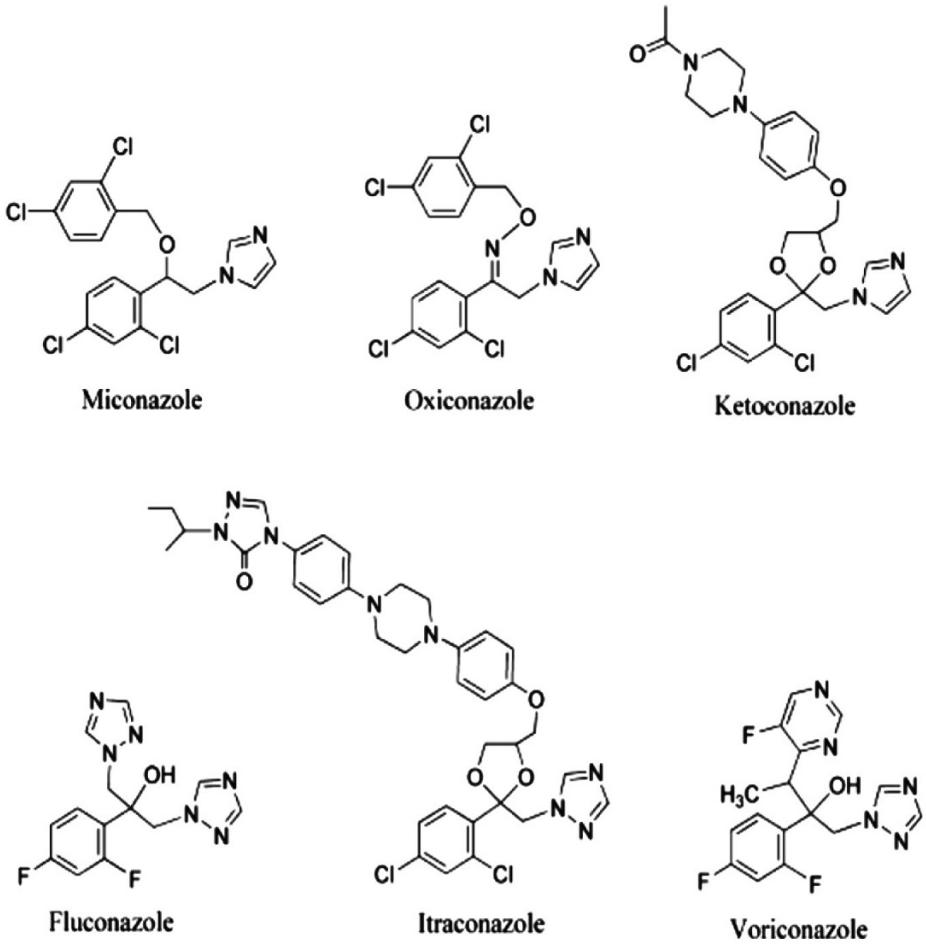


Fig. 1. Chemical structure of some clinically important antifungal azoles.

[17, 18], which has greatly reduced their efficacy. Consequently, this has led to ongoing search for new azoles.

Some azoles, like imidazole and benzimidazole derivatives (Fig. 2, L1 – 4), act as iron chelators and have been used as catalysts in oxidative reactions [19 – 22]. Based on our docking studies, we suggest these types of azoles because they can act as antifungal agents due to their ability to coordination with the heme in the active site of 14α-DM. In the present study, we report on the design and antifungal and antibacterial activities of azoles 2 – 5, 7, and 8 (Schemes 1 and 2).

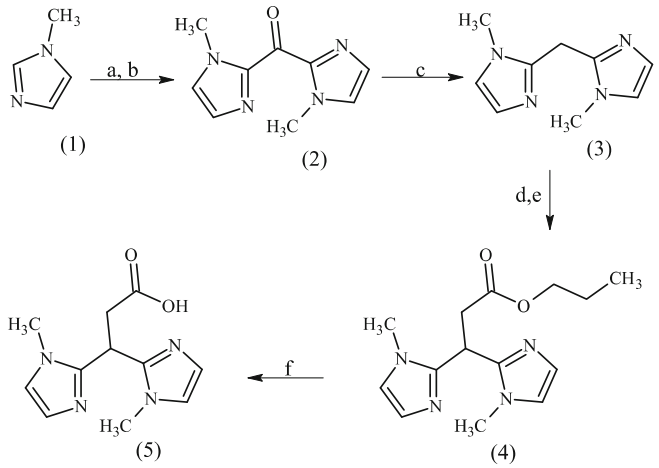
EXPERIMENTAL PART

Synthesis of Imidazole Derivatives

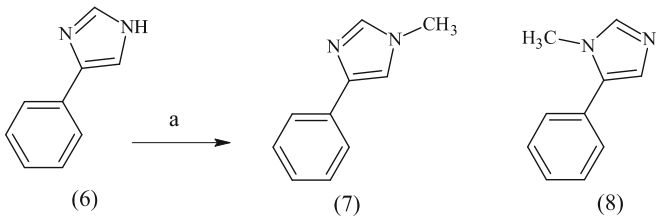
Compounds 2 – 5 (Scheme 1) were prepared using a method that had been reported by Gebbink [19 – 21]. Compounds 7 and 8 (Scheme 2) were prepared according to the literature [23]. All of the synthesized compounds were characterized by TLC, followed by IR, elemental analysis, and proton NMR.

Molecular Modeling and Docking: Software and Method

Software. The chemical structure of the desired azoles 2 – 5, 7, and 8 (Schemes 1 and 2) was built and optimized using HYPERCHEM software (Version 7, Hypercube Inc.). Conformational analysis of the compounds was performed by the semi-empirical molecular orbital calculation (PM3) method using HYPERCHEM software. Total energy gradient was calculated as a root mean square (RMS) value, until the RMS gradient was reduced to 0.01 kcal mol^{-1}. In all the conformers with energy minima, the global minimum was used in the docking calculations, and the resulted geometry was transferred to the AutoDock 4.2 program, which was developed by Arthur J. Olson Chemometrics Group [24], and docking calculations were performed using AutoDock Tools (ADT). The crystal structure of CYP51 (to 2.21Å resolution) was downloaded from the PDB bank server (PDB entry 1EA1) [26]. In the lanosterol-14α-demethylase, which was downloaded from the PDB bank server, some amino acid side chain atoms were missing. A reconstruction of the whole side chain was attempted using the Swiss-pdb viewer 4.0.1.



Scheme 1. (a) THF, TMEDA, n-BuLi, 0°C, 4h (b) Dimethylcarbamylchloride, –78°C to RT, 24 h; (c) ethylene glycol, KOH, hydrazine monohydrate, N_2, reflux, 5 h; (d) THF, n-BuLi, –78°C, 2 h; (e) propylboromoacetate, –78°C to RT, 24 h; (f) THF, KOH, RT,



Scheme 2. (a) DMSO, NaH, MeI, 80°C, 24 h.

Method. Docking studies were carried out by AutoDock 4.2 using the Lamarckian Genetic Algorithm (LGA). The algorithm uses a five-term force field-based function derived from the AMBER force field that comprises a Lennard-Jones dispersion term, a directional hydrogen bonding term, a Coulomb electrostatic potential, an entropic term, and an intermolecular pair-wise desolvation term. The ADT program, which has been released as an extension suite to the Python Molecular Viewer, was used to prepare the protein and the ligand. For the macromolecule crystal structure of lanosterol-14α-demethylase, polar hydrogen atoms were added, and then Kollman United Atom charges and atomic solvation parameters were assigned. The grid maps of the docking studies were computed using AutoGrid 4.2 that was included in the AutoDock 4.2 software. The grid center, which was centered on the active site, was obtained by trial and error and previous studies [25, 26], and 65, 65, 65 points with a grid spacing of 0.375 were calculated. The GA-LS method was adopted and defined as follows: a maximum number of 25,000,000 energy evaluations, a maximum number of 27,000 generations, and the mutation and crossover rates of 0.02 and 0.8, respectively. Pseudo-Solis and Wets parameters were used for the local search, and 300 iterations of Solis and Wets local search were imposed. The number of docking runs was set to 100. Both AutoGrid and AutoDock computations were performed on Cygwin. After docking, all generated structures were assigned to clusters based on a tolerance of 1 Å all-atom RMSD from the lowest energy structure [27].

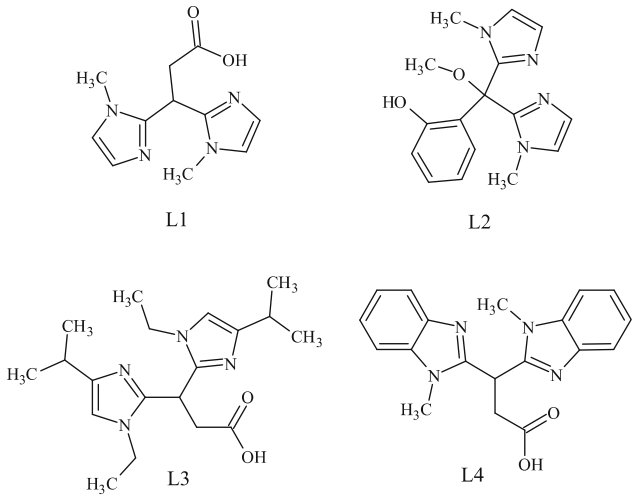


Fig. 2. Chemical structure of some azoles, ligands L_1 – L_4.

Heme – azole ring coordination, hydrogen bonding and hydrophobic interactions between docked azoles and macromolecule were analyzed using ADT (Version 1.5.4). The best docking result can be considered to be conformation with the lowest (docked) energy and rational drug – receptor interactions. In order to assign our docking methods and parameters, we docked fluconazole (Fig. 1), a drug that acts as a lanosterol-14α-demethylase inhibitor, into the active site of lanosterol-14α-demethylase and compared it to the crystalline structure of lanosterol-14α-demethylase that was inactivated by fluconazole (1EA1) [25].

Antifungal and Antibacterial Activity Evaluation

Antifungal assay. The intended compounds were tested against *Candida albicans* (ATCC10231), *Saccharomyces cervisiae* (PTCC 5052) and *Aspergillus niger* (A420) using a broth microdilution assay. The broth microdilution method was performed according to NCCLS proposed guidelines [28, 29]. All strains were sub-cultured on Sabouraud Maltose Agar (DIFCO, Becton, Dickinson, USA) growth media. For this purpose, *C. albicans* and *S. cervisiae* species were suspended in 0.9% solution of sterile NaCl; and to make the inoculums of *A. niger*, the spores were dispersed in 0.1% solution of Tween 20. The suspension turbidity of *C. albicans*, *S. cervisiae* and *A. niger* were adjusted, respectively, to 75 – 77% and 80 – 82% transmittance (*T*) using a spectrophotometer tuned at 530 nm. The suspensions were diluted 1 : 1000 for yeasts and 1 : 50 for molds in Sabouraud maltose broth (DIFCO, Becton, Dickinson, USA) for testing

by the broth micro-dilution method. Serial dilutions of tested compounds with growth medium were prepared in micro-dilution wells and 100 μl of diluted suspension was added to 100 μl of each tested compound solution. Micro-dilution trays were incubated at 35°C and examined after 48 h to determine MIC (minimum inhibitory concentration) values. Itraconazole and 2.5% solution of DMSO (Merck) were applied as positive and negative controls respectively.

Bacterial strains and antibacterial susceptibility testing. Oxacillin resistant *S. aureus* (ATCC 43300), oxacillin sensitive *S. aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) were applied for antibacterial susceptibility testing. Nutrient broth medium (Merck, Germany) with or without 4% NaCl (Merck, Germany) was used as a culture medium for *S. aureus* and *E. coli,* respectively [30]. One milliliter of an overnight test organism culture was diluted and the culture turbidity was adjusted to 0.5 McFarland. Bacterial inoculation for MIC tests was made by 100 μl of 1 : 100 dilutions of 0.5 McFarland bacterial suspensions. MICs of various compounds were determined by the micro-dilution method [31] in a 100 μl of variable substance concentrations (8 – 512 μg/ml). Microtiter plates were incubated for 24 h at 37°C and the MIC was assigned as the lowest concentration that inhibited fungal growth. Oxacillin (Sigma, USA) and streptomycin (Sigma, USA) were used as the positive control for *S. aureus* and *E. coli* respectively. Throughout all antibacterial susceptibility tests, 2.5% solution of DMSO (Merck, Germany) was applied as the negative control.

RESULTS AND DISCUSSION

Synthesis of Imidazole Derivatives

A group of imidazole derivatives including compounds 2 – 5, 7, and 8 (Schemes 1 and 2) were synthesized according to the literature in 32 – 97%. The structures of compounds were characterized by TLC followed by IR, elemental analysis, and proton NMR.

Molecular Modeling and Docking

Docking calculations were performed using AutoDock and a reconstruction of the whole side chain of the lanosterol-14α-demethylase was attempted using the Swiss-pdb viewer 4.0.1 to reconstruct some amino acid side chain atoms that were missing. In order to assign the perfect grid of each ligand, grid box values were obtained and docking was performed using the Lamarckian Genetic Algorithm (LGA) and ten independent dockings were done for each azole. The docking score, when compared to fluconazole and itraconazole, showed that these compounds docked to the active site of the enzyme. The results of docking studies, including intermolecular energy, Vdw-hb-desolv energy, electrostatic energy, total internal energy, torsional energy, unbound energy, ligand efficiency and predicted binding energy of these inhibitors into the active site, are listed in Table 1. The predicted binding energy is the sum of the intermolecular energy and the torsional free-energy penalty, wherein both can affect the mode of interaction of azoles with the enzyme 14-DM. The semi-empirical free energy force field that was used by AutoDock to evaluate conformation during docking simulation includes six pair-wise evaluations (V) and an estimate of the conformational entropy lost upon binding (?Sconf):

$$\Delta G = (V^{\text{L-L}}_{\text{bound}} - V^{\text{L-L}}_{\text{unbound}}) + (V^{\text{P-P}}_{\text{bound}} - V^{\text{P-P}}_{\text{unbound}}) + (V^{\text{P-L}}_{\text{bound}} - V^{\text{P-L}}_{\text{unbound}} + \Delta S_{\text{conf}}),$$

where L refers to the “ligand” and P refers to the “protein” in the ligand – protein docking calculation. Each of the pairwise energetic terms includes evaluations for dispersion/repulsion, hydrogen bonding, electrostatics, and desolvation wherein all of them can be affected by changes in the substituents in the azoles.

The drug – receptor interaction studies showed that all of compounds 2 – 5 with –5.33, –5.09, –6.26 and –5.46 kcal/mole binding energy, respectievly, interacted with 14α-DM by azole – heme coordination. In the azole – heme coordination, N-3 atom of one of the imidazoles binds to the

TABLE 1. Docking Results for Azoles 2 – 8 in AutoDock 4.2 Software

	Compound	Binding energy, kcal/mole	Ligand efficiency	Inhibition constant, nM	Intermol energy	Vdw-hb-desolv energy	Electrostatic energy	Total internal	Torsional energy	Unbound energy	Log*P*
2	Keton(1)	–5.33	–0.38	123.24	–5.93	–5.88	–0.05	–0.23	0.6	–0.23	–0.4
3	Methane(1)	–5.09	–0.39	186.38	–5.68	–5.68	0.0	–0.08	0.6	–0.08	–.01
4	Ester(2)	–6.26	–0.33	25.61	–8.05	–8.02	–0.03	–0.65	1.79	–0.65	–.91
5	Acid(2)	–5.46	–0.32	100.31	–6.95	–6.62	–0.33	0.18	1.49	0.18	–1.29
7	4-phenyl	–5.88	–049	48.94	–6.18	–6.17	–0.01	–0.15	0.3	–0.15	0.74
8	5-phenyl	–6.03	–0.5	37.94	–6.33	–6.33	0.0	–0.13	0.3	–0.13	0.74
	Itraconazole										

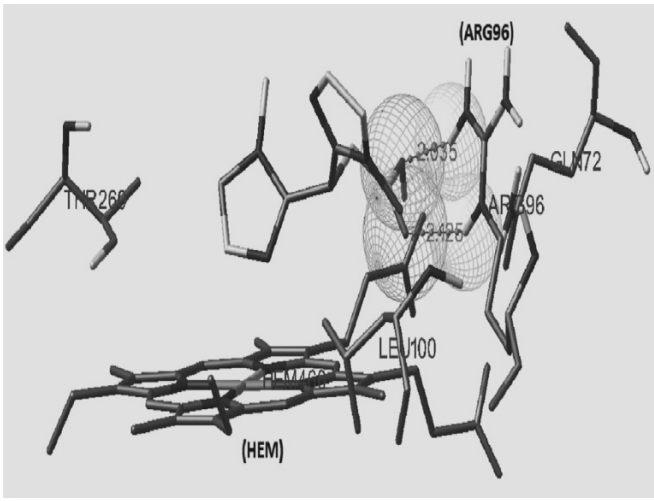


Fig. 3. Docked structure of compound 5 in the 14-alpha-demethylase model (1EA1). Compound 5 interacts with the receptor by hydrogen binding and azole – heme coordination using N-3 atom of one of the imidazoles. Hydrogen bonds are represented by wireframe spheres that have two efficient hydrogen binding (distance: 2.035 and 2.125 Å) interactions with Arg96.

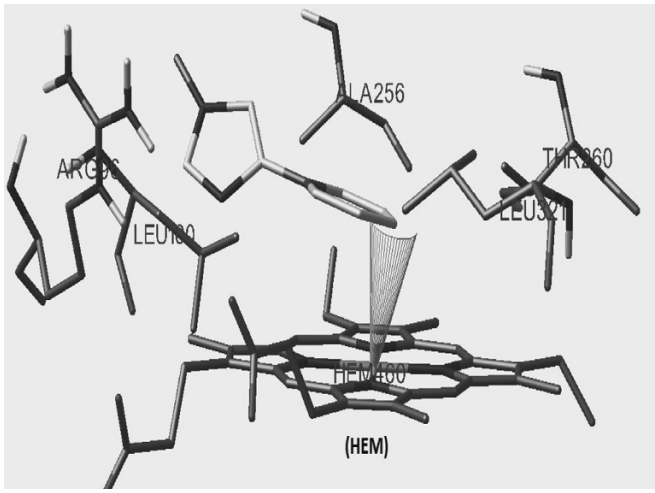


Fig. 4. Docked structure of compounds 7 (4-phenyl derivative) in the 14-alpha-demethylase model (1EA1). Compound 7 interacts with the receptor by phenyl – heme coordination (there is no imidazole – heme coordination), wherein phenyl moiety forms a π-cation interaction with the heme (shown by the cone).

heme iron atom in the active site of the enzyme (Fig. 3). In compounds 4 (ester derivative) and 5 (acid derivative), there is one and two additional hydrogen binding interactions with Arg96, respectively (Fig. 3). In compound 4, the carbonyl group acted as a hydrogen-bond acceptor site and formed a hydrogen binding (distance, 2.075 Å) with NH_2 of Arg96. In compound 5, the oxygen atom of hydroxyl and carbonyl moieties acted as two hydrogen-bond acceptor sites and formed two hydrogen bindings (distance, 2.035 and 2.125 Å) with the NH_2 and NH moieties of Arg96 respectively. These results showed that the existence of COO moiety and partition coefficient (logP) strongly affected the potency of these compounds.

The profile of compounds 7 (4-phenyl derivative) and 8 (5-phenyl derivative) interaction is different from compounds 2 – 5. In compounds 7 and 8 (Figs. 4 and 5) with –5.88 and –6.03 kcal/mole binding energy respectively, there was no imidazole – heme coordination. In these compounds, phenyl moiety formed a π-cation interaction with the heme and in compound 8, N-3 atom of imidazole acted as a hydrogen-bond acceptor site and formed hydrogen binding (distance, 2.169 Å) with the NH_2 group of Arg96. When comparing compounds 7 and 8, docking results revealed that the position of phenyl moiety strongly affected the orientation of the azole ring and subsequently the interaction of imidazole N-3 atom with the NH_2 group of Arg96 in the active site of the enzyme.

According to the docking calculation and predicted binding energy, the potency of these compounds must be in order of 4 > 8 > 7 > 5 > 2 > 3. When comparing MIC and the binding energy, there is a good and acceptable relation between

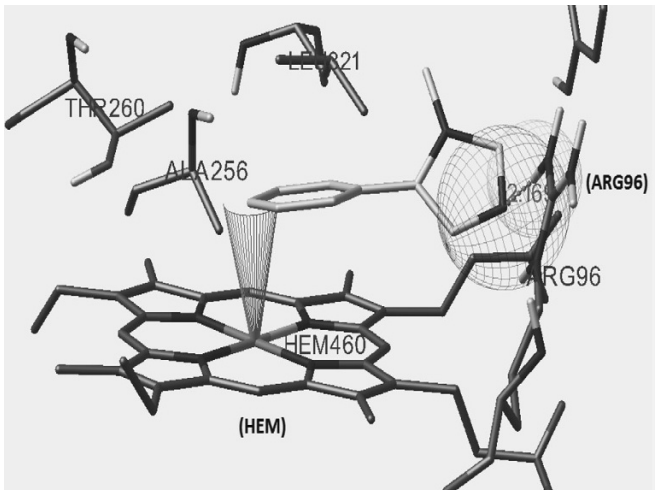


Fig. 5. Docked structure of compounds **8** (5-phenyl derivative) in the 14-alpha-demethylase model (1EA1). Compound 8 interacts with the receptor by hydrogen binding and phenyl – heme coordination (there is no imidazole – heme coordination), wherein phenyl moiety forms a π-cation interaction with the heme (shown by the cone). Hydrogen bond is represented by wireframe spheres that have one efficient hydrogen binding (distance, 2.169 A), wherein N3 atom of imidazole interacts with the NH_2 group of ARG96.

the predicted binding energy and MIC. Based on the predicted binding energy, some of these ligands (compounds 4 and 5) should be more potent; however, the experimental data did not confirm this possibly due to a low log*P* of these compounds (–0.91 and –1.29 for compounds 4 and 5, respec-

tively), which affected the drug penetration by invading the cells.

Antifungal and Antibacterial Evaluation

The in vitro antifungal activities of the synthesized compounds 2 – 5, 7 and 8 were investigated against several representative pathogenic fungi as yeast (*C. albicans*, *A. niger* and *S. cerevisiae*), (Table 2) using a broth micro-dilution assay. The MIC values were determined by comparing to itraconazole as the reference drug. The compounds were also tested against three bacterial strains, *S. aureus*, resistant *S.aureus* and *E. coli*, in which oxacillin and streptomycin were used as the standard antibiotics.

Antimicrobial evaluation (Table 2) revealed that some of the tested compounds exhibited significant antimicrobial activities against tested pathogenic fungi and bacteria. The most potent compounds 3 and 8 were active against all of the fungi and some bacteria.

In general, the MIC values of the tested compounds indicated that (i) they were more active against fungi than against bacteria and (ii) among the fungi they were mainly active against *S. cerevisiae*. Among the tested compounds, compound 3 was the most potent against *S. cerevisiae*, with a MIC value of 31.25 μg/ml. In addition, the activity of this compound against *C. albicans* and *A. niger*, with a MIC value of 500 μg/ml, was also significant. Compound 2 with a log*P* value of –0.47 was inactive against all of the fungi and bacteria in 1000 μg/ml test. Compounds 4 and 5 with log*P* values of –0.91 and –1.29 were active against *S. cerevisiae*, with MIC values of 62.5 and 250 μg/ml, respectively; however, they were inactive against *C. albicans* and *A. niger* in 1000 μg/ml test. There was low activity in these compounds, possibly due to their low partition coefficients. Compound 7 was active against *S. cerevisiae* and *A. niger*, with MIC values of 125 μg/ml and 500 μg/ml, respectively; however, it was inactive against *C. albicans* in 1000 μg/ml test. Compound 8 was active against all of the fungis, *C. albicans*, *S. cerevisiae,* and *A. niger*, with MIC values of 500, 125, and 250 μg/ml, respectively. Thus, our antifungal evaluation results showed that compounds 3 and 8 were active against the all tested fungi. Based on the antifungal results, the MIC values of the tested compounds indicated that only compounds 3 and 8 with equipotent activity were mainly active against *C. albicans*.

All of the compounds (except compound 2) were active against *S. cerevisiae* with a potency order of $3 > 4 > 7 = 8 > 5$. Among the tested compounds, compounds 3, 7 and 8 were active against *A. niger* with a potency order of $8 > 7 = 3$.

In general, the MIC values of the tested compounds indicated that their antibacterial activity in 512 μg/ml test were low against all of the bacteria. Among the bacteria, they were mainly active against *S. aureus* and resistant *S. aureus*. Among the tested compounds, compound 8 was active against all of the bacteria; *S. aureus*, resistant *S. aureus* and *E. coli* with MIC values of 64, 128, and 254 μg/ml, respectively. It was the most potent drug against *S. aureus* and resistant *S. aureus*, with MIC values of 64 and 128 μg/ml, re-

TABLE 2. Minimum Inhibitory Concentrations (MIC, μg/ml) for Antifungal and Antibacterial Activity of Synthesized Compounds (2 – 5, 7, and 8).

Compound	*Candida albicans* ATCC10231		*Saccharomyces cervisiae* PTCC 5052		*Aspergillus niger* ATCC 16404		*S. aureus* ATCC29213	Resistant *S. aureus*[a]	*Escherichia coli* ATCC25922
	24h	48h	24h	48h	24h	48h			
2	NI	NI	NI	NI	NI	NI	>512	>512	>512
3	500	NI	—	31.25	500	NI	>512	>512	>512
4	NI	NI	—	62.5	NI	NI	>512	>512	>512
5	NI	NI	—	250	NI	NI	>512	>512	>512
7	NI	NI	—	125	500	NI	>512	>512	512
8	500	500	—	125	250	500	64	128	256
Itraconazole	0.25	2	—	1	2	8			
Oxacillin				<4	>512				
Streptomycin						<4			
DMSO							Visible growth comparable with control wells	Visible growth comparable with control wells	Visible growth comparable with control wells
Control without antibiotics							Visible growth, high turbidity	Visible growth, high turbidity	Visible growth, high turbidity
Control (just medium)							No growth	No growth	No growth

spectively, wherein the activity of this compound against resistant *S. aureus* (MIC = 128 μg/ml) was superior to the reference drug, oxacillin (MIC > 512 μg/ml), and it was more active than oxacillin against *S. aureus*. In addition, the activity of this compound against *S. aureus* was comparable to that of the reference drug oxacillin. Compound 8 was promising in anti-*E. coli* activity with respect to streptomycin, with a MIC value of 256 μg/ml. Finally, we suggest that compounds 3 and 8 can act as lead compounds to finding new and potent antimicrobial agents.

CONCLUSIONS

Six analogs of 1-methylimidazole derivatives after docking studies were synthesized for evaluation as antibacterial and antifungal agents. In the docking studies, we confirmed that all these compounds interact with 14α-DM, wherein azole – heme coordination (compounds 2 – 5), π-cation interaction (compounds 7 and 8), and hydrogen binding was involved in drug – receptor interaction. Based on our molecular modeling studies, heme and Arg96 play a major role in the active site of the receptor and position of phenyl log*P*. Moreover, the existence of COO moiety in the ligand strongly affects drug – receptor interaction.

Based on the antimicrobial activity testing, compounds 3 and 8 were the most potent compounds. Compound 3 was active mainly against the fungal infection, whereas compound 8 was active against both fungal and bacterial infections, and their activity against resistant *S. aureus* was promising.

However, due to the limitations of the scoring functions, it is often difficult to establish a quantitative correlation between the calculated and experimentally derived activity values. These observations and experimental results offer some useful references in order to understand the action mechanism and molecular design performance or modification of this series of compounds as a lead compound to discover new and potent antimicrobial agents.

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