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Synthesis and antifungal activities of bisbenzazole derivatives

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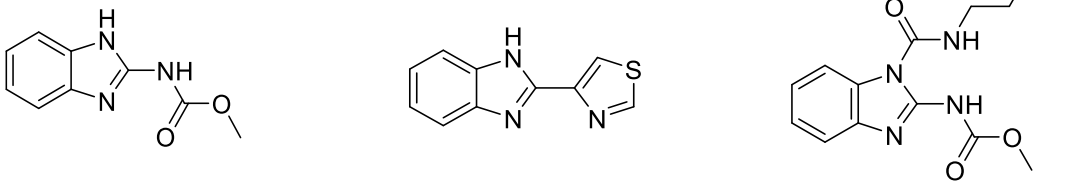
Abstract: Invasive fungal infections (IFIs) are increasing as major infectious diseases around the world, with existing medications demonstrating limited efficacy, leading to considerable morbidity and mortality due to the absence of potent antifungal agents and the emergence of serious drug resistance. In this study, a series of bisbenzazole derivatives, featuring a methyl thio linker and either 5-nitro or chloro substituent benzimidazole ring, were synthesized using straightforward and environmentally friendly reaction conditions, and characterized via ^{1}H NMR, ^{13}C NMR, and IR spectral analysis. All synthesized compounds screened *in vitro* screening for their antifungal activity against two fungal strains, namely, *C. albicans* and *C. parapsilosis*. The compounds demonstrated significant antifungal potential, particularly against *C. parapsilosis*. Furthermore, molecular docking was conducted to ascertain the affinities and potential binding poses of the compounds to the catalytic regions of the target proteins 14α-demethylase (CYP51) and secreted aspartic proteases (Sapps1p). Compounds **13** and **16** exhibited the highest affinity for CYP51, with docking scores of -6.785 and -6.923 kcal/mol, respectively. The compounds' ADMET properties were assessed in silico, revealing favorable physicochemical characteristics.

Keywords: Bisbenzazole; synthesis; antifungal activity; molecular docking.

1. Introduction

Benzimidazoles are the most prominent heterocycles with diverse biological functions[1], and their derivatives have garnered considerable interest in recent years for their versatile properties, wide spectrum of biological activity, and relatively low toxicity. Based on various literature survey benzimidazole derivatives shows that benzimidazoles to be very good antibacterial and antifungal properties[2-4]. Especially, 2-substituted benzimidazole compounds are known for their high potency as antifungal agents. Notably, several antifungal drugs, including carbendazole, thiabenzdazole and benomyl have benzimidazole-2-substituted structures (Figure 1).[5]



Carbendazole Thiabendazole Benomyl

Figure 1. Antifungal drugs with benzimidazole compounds

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It is well established that the construction of two biologically active heterocyclic units into a single compound represents a strategic approach to potential bioactive systems[6,7]. Therefore due to the wide range of interesting activities and properties of benzimidazole based compounds, the synthesis of novel benzimidazole possessing bisbenzazole hybrid compounds has been focused and studied in this research. Recent studies on bisbenzimidazole conjugates have been reported to induce their anti-proliferative and antimicrobial activities by several groups[8-16]. The above literature survey encouraged us to synthesize some new compounds containing bisbenzazole moiety hoping to obtain new compounds with potential biological activity. In our previous research, we investigated the antifungal effects of compounds with a benzimidazole and bisbenzimidazole structures. The results of the study presented significant antifungal effects. The structural variations of compounds can be classified in two regions. The first one is a benzazole rings and the second region is a linker in which there is a methyl or ethyl or propyl part at the C-2 position. The last region is a linker that carries different heteroaliphatic chain. Looking at the chemical structure of the compounds that showed stronger antifungal activity, they commonly bear hetero atom in the linker. Hence, it can be declared that C-2 of linker is a very important position interms of antifungal activity. The C-5 position of benzimidazole is also essential, and nitro or chloro substitution as electron-withdrawing group on this position significantly increases the antifungal activity. The methyl or ethyl substituents at the C-2 position of linker did not cause a meaningful difference on biological activity (Figure 2).

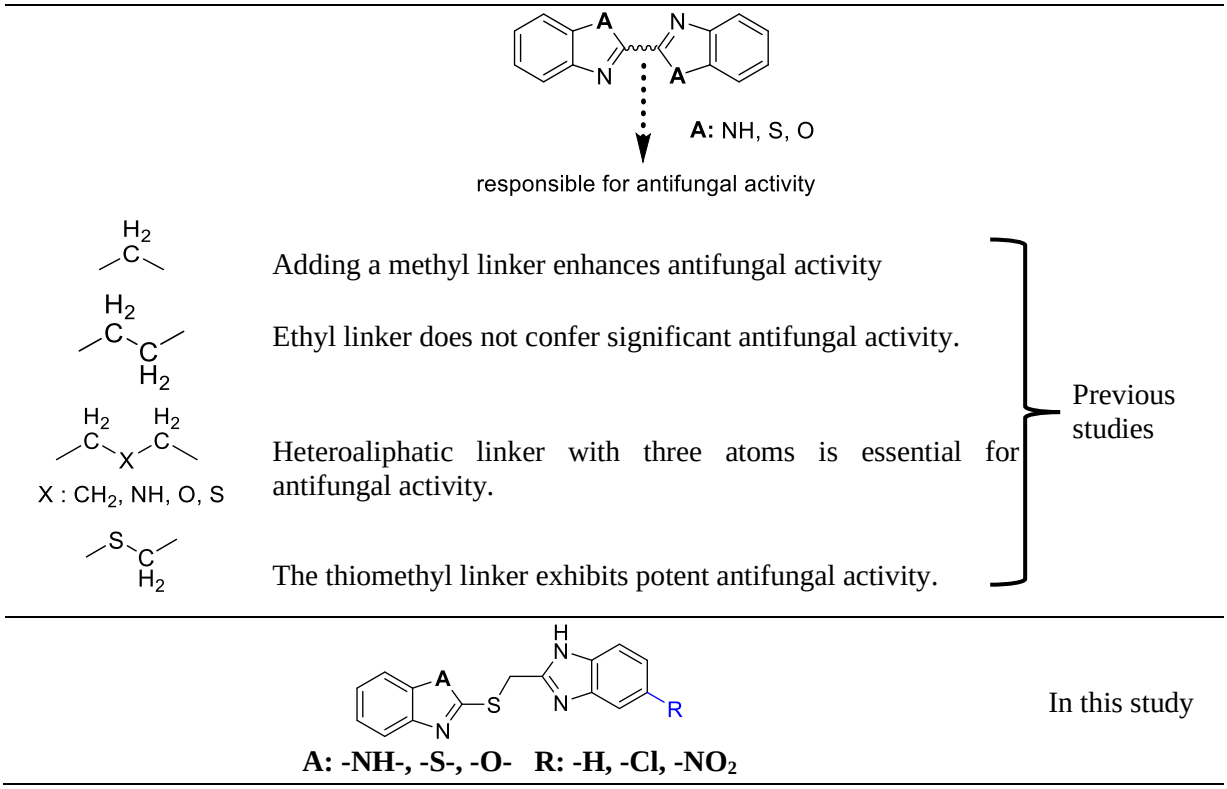


Figure 2. Design of synthesized compounds containing the bisbenzazole structure

Here, we describe the synthesis of 5-substituted bisbenzazole linked to a thiomethyl structure, where at the 2- position of benzazole rings. The structures of the synthesized derivatives were elucidated by ^{1}H NMR, ^{13}C NMR, and IR spectroscopic data. Then, we determined antifungal effects of the synthesized compounds by *in vitro* activity tests against two Candida species (*C. albicans* and *C. parapsilosis*). At the active site of 14α-demethylase, molecular docking studies of compounds were performed.

2. Experimental

2.1. Chemistry

Melting points were measured with an Electrothermal-9200 digital melting points apparatus. Melting points of the compounds were recorded on an apparatus and are uncorrected. The Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) NMR spectra were recorded by a Bruker 400 NMR (for ^{1}H NMR) and 100 NMR spectrometer (for ^{13}CNMR). ^{1}H NMR spectra and ^{13}C NMR spectra were run in deuterated dimethyl sulfoxide (DMSO–d_6), deuterated chloroform ($CDCl_3$) or deuterated methanol (MeOD-D_4). Chemical shifts (δH) are reported in parts per million (ppm, δ) relative to TMS as an internal standard. The room temperature attenuated total reflection Fourier transform infrared (FT-IR ATR) spectrum of the compounds were recorded using Varian FTS1000 FT-IR spectrometer with Diamond/ZnSe prism (4000–525 cm^{-1}; number of scans: 250; resolution: 1 cm^{-1}) in the solid. Reaction courses and product mixtures were routinely monitored by thin layer chromatography (TLC) on silica gel precoated F254 Merck plates. All chemicals used were of analytical grade and were used as received without any further purification and were obtained from Sigma-Aldrich.

*2.2. General Procedure for the Preparation of 2-(((5-Substituted-1H-benzo[d]imidazol-2-yl)methyl) thio)benzazoles[16-18] (**11-17**)*

Scheme 1 depicts the synthetic strategy for preparing the target compounds. 2-Chloroacetic acid (**4**) (1.1 eq) was added to a stirred solution of 2-mercaptobenzimidazole (**1**) / 2-mercaptobenzoxazole (**2**) / 2-mercaptobenzothiazole (**3**) (1 eq), and potassium hydroxide (1.2 eq) in methanol (20 mL). For 8 h, the reaction mixture was stirred at reflux. The solvent was removed under reduced pressure after monitoring the reaction with TLC, washed with cold water, dried, and recrystallized in ethanol to provide the intermediates (**5–7**). Compounds **5–7** (1 eq) were reacted in PPA at 150 ˚C in an oil bath for 12 h with corresponding 1,2-diaminophenyl derivatives (**8-10**) (1 eq). To obtain the precipitate, the reaction mixture was poured into ice water and neutralized by mixing with 5 M NaOH until slightly basic pH (8–9) was reached. The precipitate that formed was filtered, washed with cold water, and recrystallized with ethanol-water. Our previous study describes all synthesis procedures and results in detail.

2.3. Biological Assay

2.3.1 Antifungal Activity

Antifungal susceptibility testing was carried out using modified literature methods[19]. The microbial strains, such as *Candida albicans* (ATCC 4322), and *Candida parapsilosis* (ATTC 22019) were used for this purpose. The fungal cell inoculums were made from stock cultures grown in tryptic soy agar (TSA) at 28 ˚C for 24 h. Using sterilized saline, the microorganism suspension concentrations were adjusted according to the McFarland 0.5 turbidity tubes. The title compounds were prepared as stock solutions in DMSO at 1000 mg/mL. For antifungal activity, a modified microdilution test was used, and the experiments were run independently in duplicate.
For the antifungal activity testing, a 100 µL Tryptic Soy Broth (TSB) was added to each of the 11 wells. A 100 µL aliquot of the tested chemical solution was added to the first well, and twofold dilutions were prepared. Then, 5 µL of fungal suspension was added to each tube except the last one, which acted as the control well. A control tube containing 5 µL of the fungal suspensions alone without the tested compounds was also prepared. All plates were incubated at 28 °C for 24 h. The concentration resulting in a 50% reduction in the optical density (OD) values was compared to a reproduction control at 450 nm by spectrophotometric evaluation and defined as the MIC value. Fluconazole was used as reference drugs. The results were read visually and by measuring the OD for 24 h.

2.4. Molecular Docking and ADMET Prediction [11,20,21]

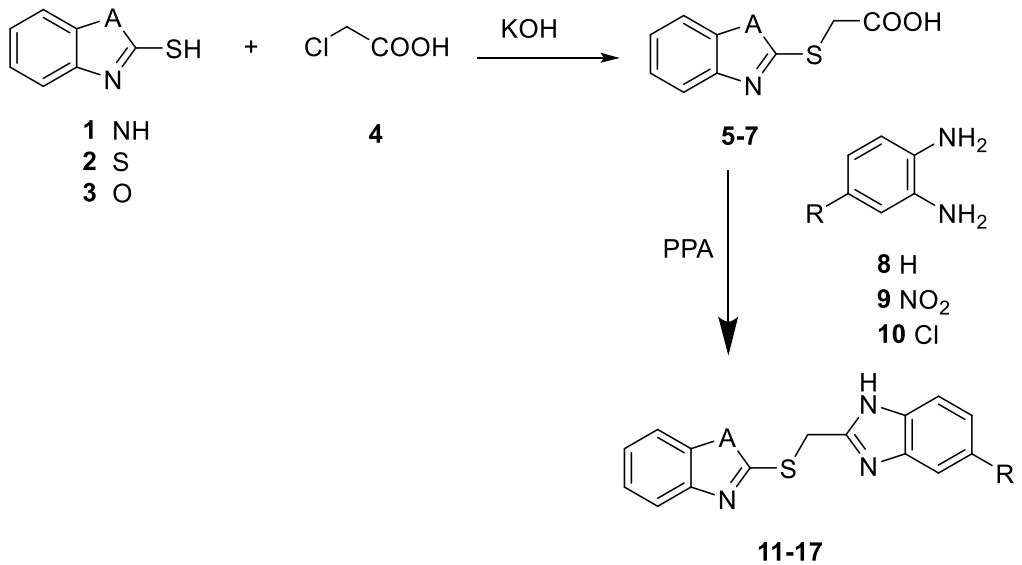
Crystal structures of target proteins (5V5Z, 7AGB) were downloaded from https://www.rcsb.org/. Structures were prepared with the Protein Preparation module of the Maestro 13.8.135 (Schrodinger) program. Water molecules in the crystal structure were removed, H atoms were added, bond order and energy minimization were carried out using the OPLS3e force field, and proteins were minimized. The co-crystallized ligands in the protein were removed, and the grid box was set to 20 A. Ligands were created and optimised using 2D Sketcher and MacroModel software. Possible tautomeric and ionisation states (pH: 7 ± 2) and enantiomers for each ligand were modelled using LigPrep of Maestro. The prepared bisbenzazol derivatives were docked to the catalytic sites of the target proteins 50 times with standard precision using Glide. The docking scores of the ligands were calculated as kcal/mol. Various physicochemical parameters of the ligands were calculated using QikProp (Maestro), and their estimated toxicity was calculated using DataWarrior 5.5.0.

3. Results and Discussion

3.1. Chemistry

The tested compounds can be classified structurally based on the nature of the substituted benzazole rings (benzimidazole, benzoxazole, and benzothiazole) as well as the length of aliphatic linkers (thiomethyl), as shown in Figure 2 and Scheme 1 contain a detailed representation of all structures. In previous studies[11], our group reported the synthesis and characteristics of compound **11**, as well as testing for other pharmacological properties.

The target molecules were synthesized in two steps as depicted in Scheme 1. First of all, as a result of the condensation reaction of the 2-chloroacetic acid (**4**) and 2-mercaptobenzazoles (**1-3**) under reflux, 2-((benzazol-2-yl)thio)acetic acid (**5-7**) was obtained. In the next and final step was carried out between compounds (**5-7**) and the appropriate 1,2-phenylendiamine derivatives (**8-10**), and the target compounds (**11-17**) were obtained. The chemical structures of the compounds were shown in Table **1**.



Scheme 1. General Procedure for Synthesis of the target Compounds **11-17**

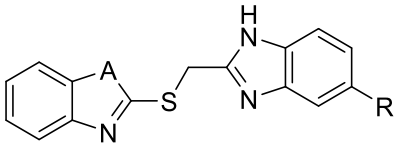
3.2. Biological Assay

3.2.1. In Vitro Antifungal Activity

The antifungal activity of the target compounds **11-17** was evaluated by an *in vitro* method against *C. albicans*, and *C. parapsilosis*. The results are listed in Table 1. When the antifungal effects of the compounds were examined, it was determined that the other compounds in the series were more effective against the Candida species included in the study compared to compound **11**. All synthesized compounds **11-17** also showed antifungal activity comparable to reference drugs (fluconazole) with MIC_{50} values of 7.81 μg/mL. Especially, compounds **16** was the most effective compounds in the series with an MIC value of 15.62 μg/mL against *C. parapsilosis*. The compound was found to be one time less effective than the reference drug fluconazole. In the series, compounds **12, 13, 14**, and **17** showed the same activity, while they were found to be four times less effective than fluconazole.
The differences in the chemical structures and the antifungal activity profiles of compounds directed us to discuss structure activity relationships (SARs) (Figure 2). In our previous study[4,11,14,16], the linker group of the benzazole rings is designed as aliphatic or heteroaliphatic. According to the antifungal activity results, it was suggested that the C-2 position of benzazoles is essential significantly increases the antifungal activity. In the previous study, it was found that the methyl or ethyl linker at the C-2 position of the ring systems did not cause a significant increase on the antifungal activity. However, the contribution of substituents on the ring to the activity had not been investigated. Therefore, in this study, instead of non-substituted benzazole, the electron-withdrawing group, the nitro and chloro groups, was used.

The synthesized compounds were derivatized on the phenyl ring with various substituents. Examining the chemical structure of the compounds (**12-17**) that showed stronger antifungal activity, they bear substituents at the C-5 position of the phenyl ring. Hence, it can be concluded that the C-5 position of the phenyl ring is very important in terms of antifungal activity. Chloro and nitro substituents at this position significantly enhance the biological activity.

Table 1. MICs of compounds, and the standards selected fungi (μg/mL)



Compound No	Compound Structure		*Candida albicans* ATCC 4322	*Candida parapsilosis ATCC* 22019
	A	R		
11	NH	H	125	62.50
12	NH	NO_2	62.50	31.25
13	S	NO_2	31.25	31.25
14	O	NO_2	62.50	31.25
15	NH	Cl	62.50	62.50
16	S	Cl	31.25	15.62
17	O	Cl	62.50	31.25
	Fluconazole		*	7.81

* All tested concentrations are active.

3.2.2. Cytotoxicity Assay

The cytotoxic effect of compounds **11-17** was evaluated against L929 cell lines. For preliminary screening, the cytotoxic bioactivity of synthesized compounds was evaluated *in vitro* against L929 cell lines with the MTT assay. To evaluate the cytotoxic potency of target compounds, the fibroblast cells were treated with the compounds at a 100 μM constant concentration. Cell viability percentages were calculated after the treatment of cells for 48 h. Preliminary dose applied, all compounds except compounds **13** and **16** showed under 50% viability. However, the compounds showed an IC_{50} value

above 100 μM, but the cell viability decreased to *55.5 and 57.1* % at the maximum dose. As a result of the calculations, it was determined that all the structures except compounds **13** and **16** had IC_{50} values below 100 μM and were found to be toxic (Table 2).

Table 2. Cell viability percentage of all compounds at 100 μM against l929 cell lines

Tested Compounds	Cell viability (%)
Control	100
11	30.4± 1.48
12	32.8± 2.56
13	55.5± 5.12
14	40.6± 1.12
15	45.7± 2.38
16	57.1± 3.68
17	35.6± 0.42

3.3. Molecular Docking and ADMET Studies

CYP51 (5V5Z) and Sapp1p (7AGB) were selected as potential targets in molecular docking studies. To make sure that the molecular docking studies were correct, the ligands were taken out of the crystal structures of 5V5Z and 7AGB. The redecok and RMSD values were then found to be 2.559 Å and 2.313 Å, respectively. We determined the affinity of bisbenzazole derivatives to these targets and their potential binding positions. The docking scores of the derivatives are given in Table 3. The docking scores of compounds **13** and compound **16**, the highest activity against CYP51, were calculated as -6,785 kcal/mol and -6,923 kcal/mol, respectively. Both compounds formed a pi-pi stacking interaction with HEM via benzothiazole rings in the active site of 5V5Z. The compounds also formed a similar pi-pi stacking interaction with benzimidazole rings and Phe233 and Phe380 (Figure 4). Compound **14** has the highest docking score against 7AGB with -4,102 kcal/mol. Compound **14** formed an H bond with Asp220 in the active site of the target protein. The compound has a salt bridge interaction with Asp32 and Asp80 and a pi-cation interaction with Tyr78 through the nitro group (Figure 5). It has been observed that the nitro group located on the benzimidazole ring is important in the compound, forming important interactions in the active site of the target protein.

Table 3. Docking scores (kcal/mol) of compounds against CYP51 (5V5Z) and Sapp1p (7AGB)

Compounds	5V5Z	7AGB
11	-4,372	-3,052
12	-5,841	-3,410
13	-6,785	-3,618
14	-6,020	-4,102
15	-6,118	-3,276
16	-6,923	-3,705
17	-6,166	-3,713

Physicochemical properties are very important for compounds to become drugs. Despite the important activities of many molecules, their poor physicochemical properties and/or toxicity have prevented their use in the clinic. Various physicochemical properties, compliance with Lipinski's rule, drug feasibility, and estimated toxicities of the compounds synthesized in the study were evaluated in silico (Table 4). All of the compounds comply with Lipinski's rule of five. None of the compounds have any predicted toxic effects (mutagenic or tumorigenic). Although the compounds do not pass into the central nervous system, their oral absorption percentage is quite high (83.92–100%).

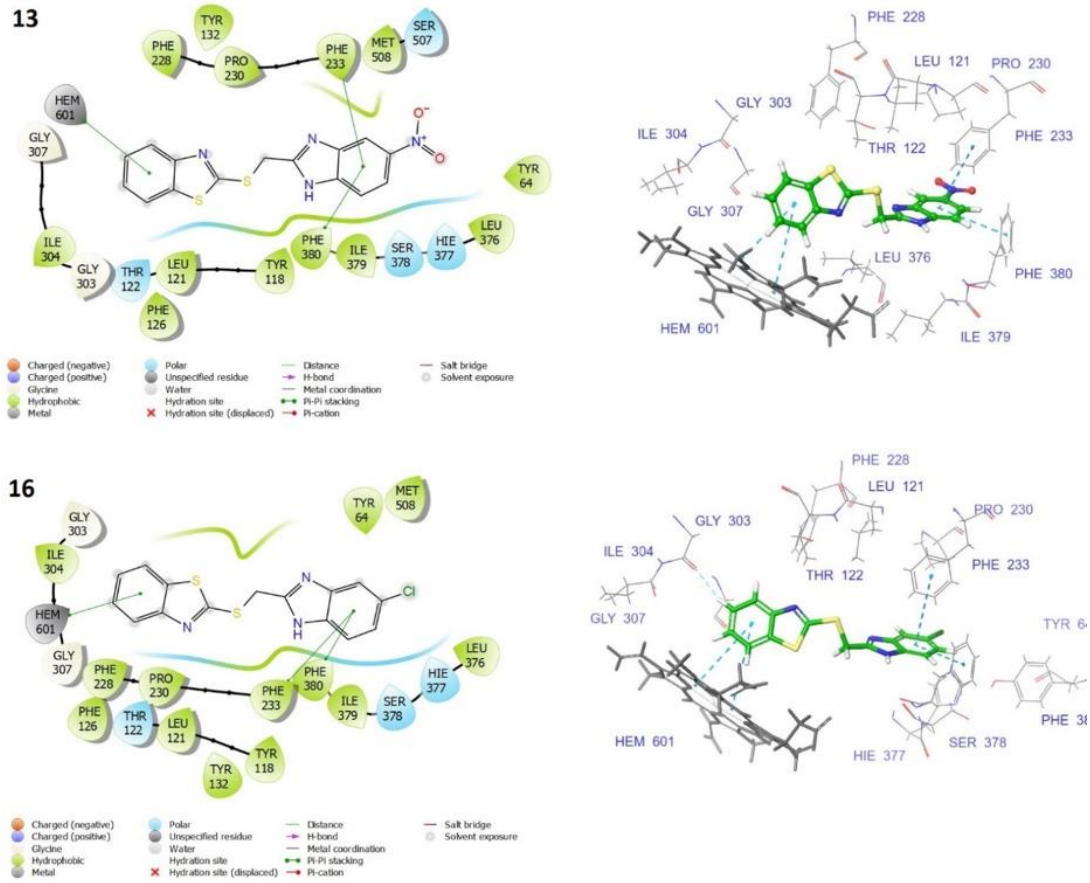


Figure 4. 2D and 3D interactions of compounds **13** and **16** in 5V5Z

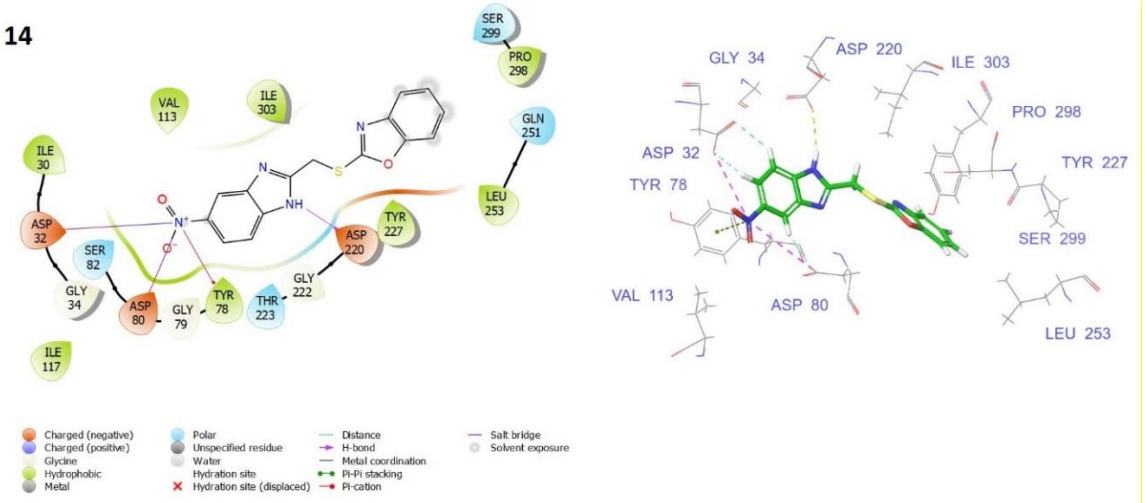


Figure 5. 2D and 3D interactions of Compound **14** in 7AGB

Table 4. Some predicted ADMET properties of compounds

Name	mol MW	logP	QPlog HERG	QPP Caco	QPlog BB	Percent Oral Absorption	CNS	Rule of Five	mutagenic	tumorigenic
11	280.34	3.48	-6.18	1535.54	-0.35	100	0	0	none	none
12	325.34	2.81	-6.09	183.75	-1.41	83.92	-2	0	none	none
13	342.39	3.43	-6.09	312.87	-1.07	91.70	-2	0	none	none
14	326.32	2.86	-6.21	253.93	-1.28	86.73	-2	0	none	none
15	314.79	3.96	-6.08	1528.14	-0.19	100	0	0	none	none
16	331.83	4.61	-6.07	2611.84	0.14	100	1	0	none	none
17	315.77	4.03	-6.17	2120.06	-0.05	100	0	0	none	none

log P: Predicted octanol/water partition coefficient (recommended value: −2.0 – 6.5). **QPlogHERG:** Predicted IC50 value for blockage of HERG (concern below −5). **QPPCaco**: Predicted apparent Caco-2 cell permeability in nm/sec. Caco-2 cells are a model for the gut-blood barrier. (<25 poor, >500 great). **CNS**: Predicted central nervous system activity on a –2 (inactive) to +2 (active) scale. **QPlogBB:** Predicted brain/blood partition coefficient (recommended value: -3.00 – 1.20). Percentage of human oral absorption (<25% is weak and > 80% is strong). **Rule of five:** Number of violations of Lipinski's rule of five (maximum is 4).

4. Conclusion

In conclusion, the objective of the current study was to synthesize and examine the antifungal activities of some new bisbenzazole derivatives with the hope of determining new structures that could be used as potent antifungal agents.

A new series of bisbenzazole derivatives (**11-17**) was synthesized and characterized with different spectroscopic methods. The target compounds (**11-17**) were evaluated for antifungal activities against two Candida species. All compounds were found to exhibit good activity against *C. glabrata* and *C. parapsilosis*. Especially, compounds **16** was found to be the most effective compounds in the series with an MIC value of 15.62 μg/ mL.

Ergosterol is the primary constituent of the cytoplasmic membrane in microorganisms and has a crucial function in preserving cell structure and permeability. Lanosterol 14α-demethylase, also known as CYP51, is an enzyme that plays a crucial role in the production of ergosterol by acting as a rate-limiting factor. The primary objective of most antifungal compounds is to impede the biosynthesis of ergosterol, thereby exerting an antifungal impact [11, 22, 23]. Azoles can inhibit the biosynthesis of ergosterol by inhibiting the enzyme lanosterol 14α-demethylase. The inhibition occurs through the assistance of an azole nucleophilic nitrogen heterocyclic ring located in the active site of lanosterol 14α-demethylase (CYP51)[24]. Secreted aspartic proteases (Saps) secreted by Candida species are specific extracellular enzymes that enable the proliferation of yeasts. These enzymes act as a barrier against attacks by the host to prevent infection [25]. Saps in yeasts break down proteins used by hosts to defend themselves, thereby increasing virulence. Saps are also effective at developing biofilm[26]. SAPs family proteins are one of the key targets for developing inhibitors that target their ability to invade tissues against candida species. *C. parapsilosis* is one of the major pathogens among Candida species. *C. parapsilosis* has SApp1p, which causes cell damage and helps the microorganism survive in the host[21]. Due to the importance of these two structures (CYP51 and Saps) in antifungal activity, the interactions of the selected and synthesized compounds were investigated through molecular docking studies. The results obtained revealed that the compounds' interactions and binding energy were consistent with their *in vitro* activities.

In addition, to establish the antifungal selectivity and safety, the cytotoxic effects of the compounds were evaluated against the L929 healthy cell line. Finally, this research provides a new chemical scaffold for the development of novel broad-spectrum antifungals, supported by *in vitro* activity results, particularly against Candida species.

Acknowledgements

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