Full Paper

Antimicrobial Activity of a New Combination System of Benzimidazole and Various Azoles

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In the present study a new series of benzimidazole derivatives bearing various (benz)azolylthio moieties were synthesized so as to investigate their antimicrobial activity. Structures of the target compounds (**5a–5i**) were confirmed by their IR, ^{1}H-NMR, ES-MS spectral data, and elemental analyses. The synthesized compounds (**5a–5i**) exhibited poor activity against bacterial strains. On the other hand, antifungal activity of the compounds against *Candida* species was very significant. Brine-Shrimp lethality assay was performed for determination of toxicity of the compounds. Compounds **5a**, **5c**, and **5d** were evaluated as non-toxic in addition to their attractive antifungal activity. However, the other compounds (**5b**, **e–i**) in the series showed toxicity to different extents.

Keywords: Antimicrobial / (Benz)azolylthio / Benzimidazole / Brine-Shrimp lethality assay / *Candida* species

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Introduction

The development of antimicrobial agents to treat infections has been one of the most important medical accomplishments of the past century. However, the progresses in medical care are threatened by a natural occurrence known as antimicrobial resistance. The increased use of antibacterial and antifungal drugs in recent years has resulted in the development of resistance to these agents [1–4] and possible microbial implications for morbidity, mortality and health care costs have become a serious fear. Even though, there are large numbers of antimicrobial drugs available for medical use, there will always be a vital need to discover new agents due to antimicrobial resistance [5, 6].

Benzimidazole is an important pharmacophore in medicinal chemistry. Recent studies have confirmed that its derivatives are effective against various strains of microorganisms [7–16]. The basis of a special interest of researchers toward benzimidazole derivatives has been 5,6-dimethylbenzimidazole, which is a fragment of cyanocobalamine (vitamin B_{12}) that is capable of inducing the growth of bacteria. However, the benzimidazole fragment and some of its derivatives suppress the bacterial growth owing to structural similarity to purine. Antibacterial ability of benzimidazoles is explained by their competition with purines resulting in inhibition of the synthesis of bacterial nucleic acids and proteins [17, 18]. Benzimidazoles exhibit not only antibacterial activity, but also antifungal activity. They can be classified as one the most important group of fungicides with systemic activity and are well-known for their pronounced ability to control a large number of fungal diseases. Thiabendazole, benomyl, carbendazim, chlorfenazole, cypendazole, debacarb, fuberidazole, mecarbinzid, and rabenzazole, which include the benzimidazole moiety, are the main examples of this fungicide class [19, 20]. In this group, the well-known fungicide thiabendazole inhibits fungal microtubular function, resulting in non-disjunction of chromosomes at cell division [21, 22].

In addition to benzimidazole, the other azole moieties such as imidazole and triazole are also important pharmacophores in medicinal chemistry field because of their chemotherapeutic value. Imidazoles and triazoles constitute two important classes of antifungal agents which are called with their names. Treatment of fungal diseases with imidazoles

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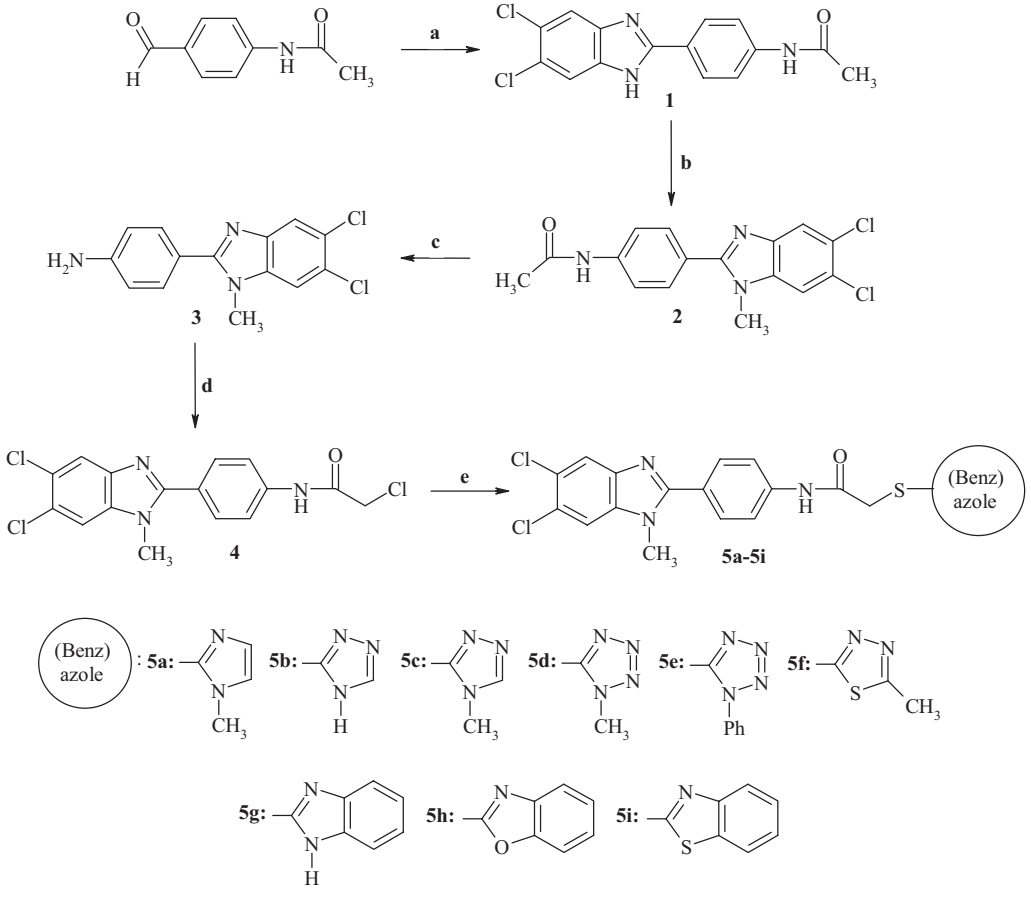
and triazoles has started with miconazole and ketoconazole and then fluconazole and itraconazole have been developed. Thus, imidazoles and triazoles have become the most attractive groups in the last two decades because they have been the most successful agents among the antifungal drugs [23, 24]. They act by competitive inhibition of the lanosterol 14α-demethylase (CYP51), [25] which is a key enzyme in sterol biosynthesis of fungi. Selective inhibition of CYP51 would cause depletion of ergosterol and accumulation of lanosterol and other 14-methyl sterols resulting in the growth inhibition of fungal cells [26]. Unfortunately, imidazoles and triazoles are fungistatic against yeasts and their broad use leads to development of resistance showing the urgent need for new and effective antifungal agents [27].

Looking at the antimicrobial importance of benzimidazole, imidazole and triazole scaffolds, we planned to synthesize a combination system that involves two different pharmacophores. Thus, in this study, some new benzimidazole derivatives bearing imidazole or triazole moieties or their bioisosters such as benzoxazole, benzothiazole, tetrazole and thiadiazole were synthesized in order to investigate their antibacterial and antifungal activity.

Results and Discussion

Chemistry

For synthesis of the target compounds (**5a–5i**) the reaction sequences outlined in Scheme 1 were followed. First of all, sodium bisulfite adduct of 4-acetylaminobenzaldehyde was prepared in dilute EtOH and reacted with 4,5-dichloro-*o*-phenylenediamine in DMF to achieve 2-(4-acetylaminophenyl)-1*H*-5,6-dichloro-benzimidazole (**1**). Secondly, *N*-methylation of **1**



Reagents and conditions; a) 1. $Na_2S_2O_5$, 80% EtOH, r.t., 0.5 h, 2. 4,5-Dichloro-*o*-phenylenediamine, DMF, 130°C, 4 h; b) K_2CO_3, CH_3I, acetone, reflux 12 h; c) Concentrated HCl, reflux, 1 h; d) TEA, $ClCH_2COCl$, benzene, ice bath and then r.t., 1 h; e) Appropriate HS-(benz)azole, K_2CO_3, acetone, reflux, 12 h.

Scheme 1. Reaction sequence of 2-[4-[2-[(benz)azolylsulfanyl]acetylamino]phenyl]-5,6-dichloro-1-methyl-1*H*-benzimidazole derivatives (**5a–5i**).

Table 1. MIC values (μg/mL) of compounds **5a–5i** against bacterial strains.

Microbial Strains	Compounds									
	5a	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**	**5i**	**Reference**
A	12.5*	6.25**	12.5*	12.5*	12.5*	12.5*	12.5*	12.5*	12.5*	12.5
B	400	200	200	200	200	200	400	200	200	12.5
C	50*	12.5**	25**	50*	50*	25**	50*	25**	12.5**	50
D	200	100	100	100	100	100	200	200	100	12.5
E	50	50	25	25	25	50	50	50	50	12.5
F	200	200	200	200	100	200	400	200	200	50
G	400	400	400	400	400	400	400	400	400	50
H	200	200	200	200	200	200	200	100	200	12.5
I	400	200	400	400	200	200	400	200	200	12.5
J	400	400	400	400	400	400	400	400	400	12.5

A: *Escherichia coli* 35218, **B**: *Escherichia coli* 25922, **C**: *Proteus vulgaris*, **D**: *Salmonella thyphimurium*, **E**: *Klebsiella pneumoniae*, **F**: *Pseudomonas aeruginosa*, **G**: *Listeria monocytogenes*, **H**: *Staphylococcus aureus*, **I**: *Enterococcus faecalis*, **J**: *Bacillus subtilis*. **Reference**: Chloramphenicol. *Equal MIC value to reference, **Lower MIC value than reference.

with CH_3I/K_2CO_3 in acetone gave 2-(4-acetylaminophenyl)-5,6-dichloro-1-methyl-1*H*-benzimidazole (**2**) which was then deacetylated with concentrated HCl to afford 2-(4-aminophenyl)-5,6-dichloro-1-methyl-1*H*-benzimidazole (**3**). Afterwards, 2-[4-(2-chloroacetylamino)-phenyl)]-5,6-dichloro-1-methyl-1*H*-benzimidazole (**4**) was prepared in benzene *via* acetylation reaction of component **3** and chloroacetyl chloride in the presence of TEA. Finally, reaction of component **4** and the appropriate (benz)azolethiol derivative in acetone in the presence of K_2CO_3 gave the title products (**5a–5i**).

Structure elucidations of the final compounds (**5a–5i**) were performed with IR, ^{1}H-NMR, and ES-MS spectroscopic methods and elemental analysis. Characteristic stretching absorptions of C=O groups and N-H bonds were observed at 1671–1681 cm^{-1} and 3325–3421 cm^{-1}, respectively. The stretching absorptions at about 1614 and 1451 cm^{-1} were assigned to C=C and C=N bonds, respectively. Disappearance of stretching absorption for S-H bond at about 2550 cm^{-1} provided evidence for the formation of the target compounds (**5a–5i**).

In the ^{1}H-NMR spectra, all of the aromatic and aliphatic protons were observed at the estimated chemical shifts. The N-H proton of the acetylamino group gave a singlet signal at 10.47–10.77 ppm. The multiplet belonging to aromatic protons of 1,4-disubstituted phenyl ring and the signals due to the protons in positions 4 and 7 of the benzimidazole ring were found at 7.69–7.82 ppm. The -$COCH_2$ and benzimidazole N-CH_3 protons singlet signals at 4.52–4.56 ppm and 3.50–3.57 ppm, respectively.

M + 1 peaks in ES-MS spectra were in agreement with the calculated molecular weight of the target compounds (**5a–5i**). Elemental analysis results for C, H, and N elements were satisfactory within ±0.4% calculated values of the compounds.

Microbiology

Table 1 presents the antibacterial activity of the compounds (**5a–5i**) against different strains of bacteria. When compared with reference drug chloramphenicol, the synthesized compounds (**5a–5i**) showed no significant antibacterial activity against *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis*, and *Listeria monocytogenes* Gram-positive bacterial strains. On the other hand, antibacterial ability of the compounds (**5a–5i**) against Gram-negative bacteria was more notable. Some of the compounds (**5c–5e**) displayed moderate activity against *Klebsiella pneumoniae* ATCC 13883. Compound **5b** was more active against *Escherichia coli* 35218 than the chloramphenicol. Other compounds (**5a**, **c–i**) in the series were found as active as the reference compound against same bacterial strain. Compounds **5a**, **5d**, **5e**, and **5g** exhibited the same antibacterial activity of the reference against *Proteus vulgaris* NRLL B-123. Furthermore, the MIC values (12.5–25 μg/mL) of compounds **5b**, **5c**, **5f**, **5h**, and **5i** were lower than that of chloramphenicol (50 μg/mL). However, all compounds (**5a–5i**) were found as inactive against other Gram-negative bacterial strains such as *Escherichia coli* 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella thyphimurium* NRRL B-4420.

In Table 2 the antifungal activity of the compounds (**5a–5i**) against different fungal strains is summarized. In contrast to their poor antibacterial activity, significant antifungal activity was displayed by most of the compounds against *Candida* yeasts. Especially *Candida albicans* was more sensitive to the tested compounds (**5a–5i**). Most of the compounds **(5a–5f**, **5h)** in the series exhibited better antifungal activity than reference drug ketoconazole against *C. albicans*. Compounds **5g** and **5i** were found to be as active as the reference (MIC = 50 μg/mL) against this fungal strain. Only **5g** and **5i** exhibited lower antifungal activity than ketoconazole

Table 2. MIC values (μg/mL) of compounds **5a–5i** against fungal strains.

Microbial Strains	Compounds									
	5a	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**	**5i**	**Reference**
A	25**	25**	25**	25**	25**	25**	50*	25**	50*	50
B	25*	25*	25*	25*	25*	25*	25*	25*	25*	25
C	50*	50*	50*	50*	50*	50*	100	100	50*	50

A: *Candida albicans*, **B**: *Candida globrata*, **C**: *Candida tropicalis*. **Reference**: Ketaconazole.*Equal MIC value to reference,**Lower MIC value than reference.

against *Candida tropicalis*. Antifungal activities (MIC = 50 μg/mL) of the other compounds (**5a–f**, **h**) in the series against this fungal strain were the same of ketoconazole. All of the compounds (**5a–5i**) indicated the same MIC value (25 μg/mL) of the reference against *Candida globrata*.

The successful development of a new drug depends on a number of criteria that have to be met. For example, in addition to intrinsic activity, the drug must be able to reach its target and should not produce toxic effect. Hence, toxicity of the synthesized compounds **(5a–5i)**, which have significant antifungal activity, was needed to be investigated. For this purpose, Brine-Shrimp lethality assay, which has been regarded as a useful method for preliminary evaluation of toxicity and used for the establishing of fungal toxins, plant extract toxicity, heavy metals, cyanobacteria toxins, pesticides, cytotoxicity testing of dental materials [28], natural and synthetic organic compounds [29], was performed. Such toxicity test results show a good correlation with rodent and human acute oral toxicity data. Likewise, the predictive screening potential of the aquatic invertebrate tests for acute oral toxicity in man, including Brine-Shrimp toxicity test, are slightly better than the rat tests for the reference compounds [30].

Toxicity test results were analyzed by the LC_{50} computer program (Trimmed Spearman-Karber Method, Version 1.5) so as to calculate LC_{50} values and 95% confidence intervals [31] (Table 3). After evaluating MIC and LC_{50} values of the compounds (**5a–5i**) together, the **5a**, **5c**, and **5d** ($LC_{50} > 1000$ μg/mL) were determined as non-toxic. On the other hand, the other compounds (**5b**, **e–i**) showed toxicity to different extents. Compound **5b** ($LC_{50} = 103.11$ μg/L) was found to be harmful. Compound **5e** ($LC_{50} = 33.49$ μg/L), **5f** ($LC_{50} = 51.43$ μg/L), and **5h** ($LC_{50} = 71.73$ μg/L) were established as toxic. Compounds **5g** and **5i** ($LC_{50} < 7.8$ μg/L) were designated as very toxic. The reference drug ketoconazole was also assayed in toxicity test and it was assessed as non-toxic ($LC_{50} = 793.70$ μg/L). Compounds **5a**, **c**, **d**, and ketoconazole found to be as non-toxic, calculation of higher LC_{50} for the tested compounds (**5a**, **c**, **d**) than reference was an important finding which indicates that toxicity level of these three compounds is weaker than that of the reference.

Comparison of the results of toxicity, antibacterial and antifungal activity tests shows that the tested compounds (**5a–5i**) have weak antibacterial activity against both Gram-negative and Gram-positive bacterial strains. On the other hand, significant inhibitory activity against *Candida* yeasts points out the antifungal potential of the compounds. Compounds **5a**, **5c**, and **5d** are the most noteworthy in the series because they display antifungal activity at non-toxic concentrations. Hence, the antifungal activity of the compounds is not due to their general toxicity effect but can be ascribed to the selectivity of their antifungal properties.

Table 3. Brine-Shrimp toxicity results of compounds **5a–5i**.

Compound	LC_{50} (μg/mL)	Lower 95% limit	Upper 95% limit	Toxicity*
5a	>1000.00	–	–	Non-toxic
5b	103.11	44.06	241.30	Harmful
5c	>1000.00	–	–	Non-toxic
5d	>1000.00	–	–	Non-toxic
5e	33.49	9.48	188.88	Toxic
5f	51.43	23.19	114.06	Toxic
5g	<7.81	–	–	Very toxic
5h	71.73	13.33	385.92	Toxic
5i	<7.81	–	–	Very toxic
Ketocanazole	793.70	566.86	1111.31	Non-toxic

*Evaluated by comparing with MIC values of the compounds against *Candida* species.

In terms of structure-activity relationships (SARs), the substitution pattern was explored using various bioisosteric (benz)azolylthio moieties arising from 1-methyl-1*H*-imidazole-2-thiol, 1,2,4-4*H*-triazole-3-thiol, 1-methyl-1,2,4-4*H*-triazole-3-thiol, 1-methyl-1,2,3,4-1*H*-tetrazole-5-thiol, 1-phenyl-1,2,3,4-1*H*-tetrazole-5-thiol, 5-methyl-1,2,4-thiadiazole-3-thiol, 1*H*-benzimidazole-2-thiol, benzoxazole-2-thiol, and benzothiazole-2-thiol in 2-[4-(acetylamino)phenyl]-5,6-dichloro-1-methyl-1*H*-benzimidazole main substructure. Thus, determination of contribution of the various bioisosteric (benz)azolylthio moieties to antibacterial and/or antifungal activity and evaluation of SARs were planned. However, SARs could not be discussed due to poor antibacterial activity of the compounds (**5a–5i**). Moreover, observation of very similar antifungal activity displayed by the compounds (**5a–5i**) indicates that there is no important difference between contributions of azolylthio moieties to antifungal activity and makes consideration of the SARs very difficult. Hence, it can be assumed that antifungal activity of the compounds (**5a–5i**) is related to their general structural characteristics provided by both bioisosteric azolylthio moieties and 2-[4-(acetylamino)phenyl]-5,6-dichloro-1-methyl-1*H*-benzimidazole substructure.

Contrary to SARs, a relationship between structural properties of the compounds (**5a–5i**) and their toxicological effect is easily understood because the tested compounds (**5a–5i**) have indicated toxicity to different extents, which can be the result of difference between structural properties. In the series, the most toxic compounds **5e**, **5g**, **5h**, and **5i** contain 1-phenyl-1*H*-tetrazol-5yl-thio, 1*H*-benzimidazol-2-ylthio, benzoxazol-2-ylthio and benzothiazol-2-ylthio moieties which enhance lipophilic character of such derivatives. It is known that there is a relationship between lipophilicity and toxicity. In general, once the lipophilicity increases, a rise in the toxicity also occurs. Depending on this respect, it may be concluded that higher toxic effect of the compounds **5e**, **5g–i** than the other derivatives is related to their increased lipophilicity. On the other hand, compounds **5b** and **5f** are not as lipophilic as the **5e**, **5g**, **5h**, and **5i,** but they are the other toxic derivatives in the series. Hence, an additional reason to high lipophilicity, which may be responsible for the toxicity, should be clarified. If the structures of toxic derivatives (**5b** and **5h**) are compared with those of the non-toxic derivatives (**5a**, **5c**, and **5d**), the most evident difference consist of presence of *N*-methyl groups of the azole rings in **5a**, **5c**, and **5d**. It has been reported that, in general, the presence of electron-donating groups, such as methyl, seems to reduce the toxicity, whereas electron-withdrawing substituents produce higher toxic effect [32]. This approach has been supported by the results of our study in which the *N*-methyl substituted compounds **5a**, **c**, **d** displayed no toxicity and can be explained by the effect of high electron density, which causes more difficult diffusion through the cell membrane [33]. In the series, electron density of the non-toxic compounds **5a**, **c**, **d** is higher than that of the toxic ones because there is an electron flow from electron donating methyl group to electron withdrawing nitrogen atom of the imidazole, triazole or tetrazole ring. Thus, toxicity loss in the *N*-methyl substituted compounds **5a**, **5c**, and **5d** may be due to retardation in intracellular transport because of high electron density.

Conclusion

Nowadays growing resistance of pathogens against current antibacterial and antifungal agents is a common and important medical problem and research on new compounds against these pathogens are needed. Hence, we have synthesized some novel benzimidazole-azole derivatives and screened for their probable antimicrobial activity. Results of this study have indicated that most of the synthesized compounds are very effective against *Candida* species. Furthermore, compounds **5a**, **c**, **d** have been recognized as non-toxic and this has improved their chemotherapeutic value. It has been observed that non-toxicity of the compounds **5a**, **5c**, and **5d** may be related to their low liphophilicity and high electron density. However, there is no relationship between these physicochemical properties and antimicrobial potency which can be a result of selective antifungal effect of the compounds (**5a–5i**).

Consequently, significant antifungal activity displayed by a novel combination system of benzimidazole and the other bioisosteric (benz)azole rings have encouraged us to make some modifications on basic structure of obtained compounds (**5a–5i**) to achieve more active derivatives in ongoing studies. Moreover, we believe that findings of the present study will have a good impact on medicinal chemists to synthesize similar compounds which will probably indicate greater antifungal activity.

Materials and Methods

Chemistry

Melting points (m.p.) of the final compounds (**5a–5i**) were determined in open capillaries on an Electrothermal 9001 Digital Melting Point Apparatus and are uncorrected. The purity of the compounds was routinely checked by thin layer chromatography (TLC) using silica gel 60. IR spectra were recorded on a Shimadzu 8400 FTIR spectrometer as KBr pellets. ^{1}H-NMR spectra were recorded on a Bruker UltraShield 500 MHz spectrometer in DMSO-d_6. MS data were obtained on an Agilent 1100 Series LC/MSD Trap VL&SL spectrometer. Elemental analyses (C, H, and N) were determined on a Perkin Elmer analyzer.

The starting compounds **1** and **2** were synthesized according to previously described methods [9, 34].

2-(4-Aminophenyl)-5,6-dichloro-1-methyl-1H-benzimidazole ***(3)***

A solution of **2** (20.0g, 59.88 mmol) in conc. aq. HCl (50 mL) was refluxed for 1 h and then allowed to cool and poured into ice-water (50 mL). Neutralization with 10% aq. NaOH resulted in the formation of a solid which was collected by filtration, washed several times with cold water, and then recrystallized from ethanol to give **3**. Yield 86%. M.p. 201°C. IR (KBr) ν_{max} (cm^{-1}): 3349–3332 (N-H), 1684 (C=O), 1607–1451 (C=C and C=N).

2-[4-(2-Chloroacetylamino)-phenyl)]-5,6-dichloro-1-methyl-1H-benzimidazole ***(4)***

Triethylamine (8.5 mL, 60 mmol) was added to a solution of **3** (14.6 g, 50 mmol) in benzene (100 mL). A solution of chloroacetyl chloride (4.8 mL, 60 mmol) in benzene (10 mL) was added dropwise under vigorous stirring to the above mixture maintaining the temperature below 5°C (ice bath). The resulting mixture was stirred for 1 h at room temperature and then evaporated to dryness. The residue was recrystallized from ethanol to give **4**. Yield 73%. M.p. 250–252°C (decomp.). IR (KBr) ν_{max} (cm^{-1}): 3336 (N-H), 1688 (C=O), 1602–1456 (C=C and C=N).

General synthesis procedure for target compounds ***(5a-5i)***

A mixture of **4** (737 mg, 2.0 mmol), the appropriate (benz)azolethiol (2.0 mmol), and K_2CO_3 (276 mg, 2.0 mmol) in acetone was refluxed for 12 h. The residue was washed with cold water and recrystallized from ethanol to give **5a–5i**.

5,6-Dichloro-1-methyl-2-[4-[2-[(1-methyl-1H-imidazole-2-yl)sulfanyl]acetylamino]phenyl]-1H-benzimidazole ***(5a)***

Yield 77%. M.p. 230°C. IR (KBr) ν_{max} (cm^{-1}): 3329 (N-H), 1674 (C=O), 1611–1452 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 2.97 (s, 3H, imidazole N-CH_3), 3.57 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 4.52 (s, 2H, CO-CH_2), 7.71–7.79 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 8.01 (d, H, J = 7.3 Hz, imidazole C_3-H), 8.17 (d, H, J = 7.3 Hz, imidazole C_2-H), 10.71 (s, H, NH-CO). ES-S (m/z): M + 1: 447.5. Anal. calcd. for $C_{20}H_{17}Cl_2N_5OS$ (446.36): C, 53.82; H, 3.84; N, 16.69. Found: C, 53.94; H, 3.85; N, 16.72.

5,6-Dichloro-1-methyl-2-[4-[2-[(4H-1,2,4-triazole-3-yl)sulfanyl]acetylamino]phenyl]-1H-benzimidazole ***(5b)***

Yield 64%. M.p. 178°C. IR (KBr) ν_{max} (cm^{-1}): 3421–3325 (N-H), 1671 (C=O), 1608–1453 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.54 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 4.53 (s, 2H, CO-CH_2), 7.69–7.77 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 8.53 (s, H, triazole C_2-H), 10.47 (s, H, NH-CO). 12.91 (br, H, triazole N-H). ES-MS (m/z): M + 1: 434.3. Anal. calcd. for $C_{18}H_{14}Cl_2N_6OS$ (433.32): C, 49.89; H, 3.26; N, 19.39. Found: C, 49.96; H, 3.25; N, 19.42.

5,6-Dichloro-1-methyl-2-[4-[2-[(4-methyl-4H-1,2,4-triazole-3-yl)sulfanyl]acetylamino]phenyl]-1H-benzimidazole ***(5c)***

Yield 73%. M.p. 268°C. IR (KBr) ν_{max} (cm^{-1}): 3332 (N-H), 1678 (C=O), 1610–1451 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.54 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 3.65 (s, 3H, triazole N-CH_3), 4.53 (s, 2H, CO-CH_2), 7.73–7.80 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 8.58 (s, H, triazole C_2-H), 10.55 (s, H, NH-CO). ES-MS (m/z): M + 1: 448.4. Anal. calcd. for $C_{19}H_{16}Cl_2N_6OS$ (447.35): C, 51.01; H, 3.61; N, 18.79. Found: C, 51.15; H, 3.62; N, 18.65.

5,6-Dichloro-1-methyl-2-[4-[2-[(1-methy-1H-1,2,3,4-tetrazole-5-yl)sulfanyl]acetylamino]phenyl]-1H-benzimidazole ***(5d)***

Yield 81%. M.p. 279°C. IR (KBr) ν_{max} (cm^{-1}): 3328 (N-H), 1673 (C=O), 1610–1466 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.52 (s, 3H, 5,6-dichlorobenzimidazole), 4.01 (s, 3H, tetrazole N-CH_3), 4.56 (s, 2H, CO-CH_2), 7.73–7.80 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 10.62 (s, H, NH-CO). ES-MS (m/z): M + 1: 449.5. Anal. calcd. for $C_{18}H_{15}Cl_2N_7OS$ (448.34): C, 48.22; H, 3.37; N, 21.87. Found: C, 48.11; H, 3.38; N, 21.85.

5,6-Dichloro-[4-[2-[(1-phenyl-1H-1,2,3,4-tetrazole-5-yl)sulfanyl]acetylamino]phenyl]-1-methyl-2-1H-benzimidazole ***(5e)***

Yield 72%. M.p. 287°C. IR (KBr) ν_{max} (cm^{-1}): 3331 (N-H), 1674 (C=O), 1614–1485 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.50 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 4.54 (s, 2H, CO-CH_2), 7.59–7.64 (m, 3H, tetrazole N-Ph $C_{3,4,5}$-H), 7.73–7.80 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 7.99 (d, 2H, J = 8.6 Hz, tetrazole N-Ph $C_{2,6}$-H), 10.68 (s, H, NH-CO). ES-MS (m/z): M + 1: 511.4. Anal. calcd. for $C_{23}H_{17}Cl_2N_7OS$ (510.41): C, 54.12; H, 3.36; N, 19.21. Found: C, 54.05; H, 3.34; N, 19.15.

5,6-Dichloro-1-methyl-2-[4-[2-[(5-methyl-1,2,4-thiadiazole-3-yl)sulfanyl]acetylamino]phenyl]-1H-benzimidazole ***(5f)***

Yield 78%. M.p. 221°C. IR (KBr) ν_{max} (cm^{-1}): 3335 (N-H), 1672 (C=O), 1611–1489 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 2.69 (s, 3H, thiadiazole C_2-CH_3), 3.52 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 4.53 (s, 2H, CO-CH_2), 7.74–7.82 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 10.62 (s, H, NH-CO). ES-MS (m/z): M + 1: 465.6.

Anal. calcd. for $C_{19}H_{15}Cl_2N_5OS_2$ (464.40): C, 49.14; H, 3.26; N, 15.08. Found: C, 49.05; H, 3.26; N, 15.11.

5,6-Dichloro-2-[4-[2-[(1H-benzimidazole-2-yl)sulfanyl]acetylamino]phenyl]-1-methyl-1H-benzimidazole ***(5g)***

Yield 82%. M.p. 197°C. IR (KBr) ν_{max} (cm^{-1}): 3408–3346 (N-H), 1677 (C=O), 1608–1452 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.53 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 4.54 (s, 2H, CO-CH_2), 7.30–7.33 (m, 2H, benzimidazole $C_{3,4}$-H), 7.72–7.82 (s, 8H, benzimidazole $C_{2,5}$-H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 10.77 (s, H, NH-CO) 12.85 (br, H, benzimidazole N-H). ES-MS (*m*/*z*): M + 1: 483.5. Anal. calcd. for $C_{23}H_{17}Cl_2N_5OS$ (482.39): C, 57.27; H, 3.55; N, 14.52. Found: C, 57.21; H, 3.54; N, 14.55.

5,6-Dichloro-2-[4-[2-[(benzoxazole-2-yl)sulfanyl]acetylamino]phenyl]-1-methyl-1H-benzimidazole ***(5h)***

Yield 82%. M.p. 241°C. IR (KBr) ν_{max} (cm^{-1}): 3330 (N-H), 1676 (C=O), 1604–1454 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.52 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 4.56 (s, 2H, CO-CH_2), 7.35–7.39 (m, 2H, benzoxazole $C_{3,4}$-H), 7.64–7.69 (m, 2H, benzoxazole $C_{2,5}$-H), 7.72–7.80 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 10.68 (s, H, NH-CO). ES-MS (*m*/*z*): M + 1: 484.4. Anal. calcd. for $C_{23}H_{16}Cl_2N_4O_2S$ (483.38): C, 57.15; H, 3.34; N, 11.59. Found: C, 57.35; H, 3.33; N, 11.55.

5,6-Dichloro-2-[4-[2-[(Benzothiazole-2-yl)sulfanyl]acetylamino]phenyl]-1-methyl-1H-benzimidazole ***(5i)***

Yield 84%. M.p. 204°C. IR (KBr) ν_{max} (cm^{-1}): 3347 (N-H), 1681 (C=O), 1609–1452 (C=C and C=N). ^{1}H-NMR (500 MHz) (DMSO-d_6) δ (ppm): 3.52 (s, 3H, 5,6-dichlorobenzimidazole N-CH_3), 4.55 (s, 2H, CO-CH_2), 7.25–7.32 (m, 2H, benzothiazole $C_{3,4}$-H), 7.73–7.81 (m, 6H, 5,6-dichlorobenzimidazole and 1,4-disubstituted benzene Ar-H), 7.86 (d, 2H, J = 8.1 Hz benzothiazole C_5-H), 8.05 (d, 2H, J = 8.0 Hz benzothiazole C_2-H), 10.68 (s, H, NH-CO). ES-MS (*m*/*z*): M + 1: 500.5. Anal. calcd. for $C_{23}H_{16}Cl_2N_4OS_2$ (499.44): C, 55.31; H, 3.23; N, 11.22. Found: C, 55.15; H, 3.24; N, 11.19.

Microbiology

Final products were tested for their *in-vitro* growth inhibitory activity against human pathogenic as Gram-positive bacteria; *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* and *Listeria monocytogenes* (obtained from Faculty of Pharmacy Anadolu University, Eskisehir, Turkey), as Gram-negative bacteria; *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Escerichia coli* ATCC 35218, *E. coli* ATCC 25922, *Salmonella thyphimurium* NRRL B-4420 and *Proteus vulgaris* NRLL B-123 and yeast as *Candida albicans*, *C. tropicalis* and *C. globrata* ATCC 36583 (obtained from Faculty of Medicine Osmangazi University, Eskisehir, Turkey). Chloramphenicol and ketoconazole were used as control drugs.

The synthesized compounds (**5a–5i**) were tested for their toxicity by applying Brine-Shrimp lethality assay. Fresh eggs of Brine-Shrimp (*Artemia salina*), sold as a fish food, were purchased from the local pet shop, Eskisehir/Turkey. Ketoconazole was used in the assay to compare toxicity levels of the synthesized compounds (**5a–5i**) with that of reference antifungal agent.

Antimicrobial assay

Antimicrobial activity assay was performed according to CLSI reference M7-A7 broth microdilution method as described in previous study [35]. Twice MIC readings were carried out for each chemical agent. The compounds (**5a–5i**) were dissolved in DMSO for antibacterial and antimycotic assays. Further dilutions of the compounds (**5a–5i**) and standard drugs in test medium were prepared at the required quantities of 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 μg/mL concentrations with Mueller-Hinton broth and Sabouroud dextrose broth. In order to ensure that the solvent *per se* had no effect on bacteria or yeast growth, a control test was also performed containing inoculated broth supplemented with only DMSO at the same dilutions used in our experiments and found inactive in culture medium.

Brine-Shrimp lethality assay

Brine-Shrimp toxicity assay was used for determination of cytotoxicity levels of the synthesized compounds (**5a–5i**). Each test compound was dissolved in DMSO to obtain the stock concentration of 1000 μg/mL and then stock solution was diluted to various concentrations (1000–7.8125 μg/mL). In order to prevent the toxicity results from possible false effects originated from DMSO's toxicity, stock solutions of the compounds (**5a–5i**) were prepared according to suggested volume range by dissolving 1 mg of test compound in 10 μL DMSO and completing to 1000 μL with artificial seawater [29]. Pure DMSO was used as a positive control for the toxicity assay. The eggs of Brine-Shrimp hatched in a conical flask containing 300 ml artificial seawater made by dissolving a commercial marine salt in deionized water. The flasks were well aerated with the aid of an air pump, and kept in a water bath at 25–30°C. The larvae hatched within 48 h. Ten larvae were transferred with pipetter into each vial containing test compound and artificial sea water. A check count was performed after 24 h of exposure at room temperature and the number of dead larvae, exhibiting no internal or external movement during several seconds of observation, was noted. Three independent

experiments were performed for each concentration of compounds (**5a–5i**).

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