European Journal of Medicinal Chemistry 45 (2010) 3293–3298

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Antimicrobial activity and a SAR study of some novel benzimidazole derivatives bearing hydrazone moiety

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ARTICLE INFO

Article history:
Received 10 December 2009
Received in revised form
12 April 2010
Accepted 13 April 2010
Available online 18 April 2010

Keywords:
Benzimidazole
Hydrazone
Antifungal activity
Antimicrobial activity
Structure–activity relationship
Brine-shrimp lethality assay

ABSTRACT

In this study 12 novel benzimidazole compounds bearing hydrazone moiety were synthesized in order to investigate their possible antibacterial and antifungal activity. Structures of the synthesized compounds were elucidated by spectral data. Six different gram-negative and four different gram-positive bacterial strains were used in antibacterial activity tests. Antifungal activity tests were also performed against three different fungal strains. Most of the test compounds found to be significantly effective against *Proteus vulgaris*, *Staphylococcus typhimurium*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* gram-negative bacterial strains. A structure–activity relationship (SAR) study including some electronic parameters was carried out and a connection between antibacterial activity and electronic properties of the target compounds was determined. Toxicity of the most effective compounds was established by performing Brine–Shrimp lethality assay.

1. Introduction

Infectious microbial diseases remain pressing problems worldwide, because resistance to a number of antimicrobial agents (β-lactam antibiotics, macrolides, quinolones, and vancomycin) among variety of clinically significant species of microorganisms has become an important global health problem [1]. One way to battle with this challenge is the conscious usage of the currently marketed antibiotics; the other is the development of novel antimicrobial agents [2]. Hence, there will always be a vital need to discover new chemotherapeutic agents to avert the emergence of resistance and ideally shorten the duration of therapy.

Benzimidazole is an important pharmacophore and privileged structure in medicinal chemistry. Literature survey shows that among the benzimidazole derivatives, 2-substituted ones are found to be pharmacologically more potent and hence the design and synthesis of 2-substituted benzimidazoles are the potential area of research [3,4]. Extensive biochemical and pharmacological studies have confirmed that its derivatives are effective against various strains of microorganisms [5–15]. The reason for a special interest of researchers toward benzimidazole derivatives has been 5,6-dimethylbenzimidazole which is a constituent of naturally occurring vitamin B_{12} [16]. Although vitamin B_{12} is capable of inducing the growth of bacteria, the benzimidazole component and some of its derivatives repress the bacterial growth. Due to the 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].

In addition to their antibacterial activity, benzimidazole derivatives possess antifungal activity. They can be classified as 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. Benomyl, thiabendazole and thiophnate methyl are some main examples of this fungicide class. Owing to their systemic activity, they can help to control some infectious microbial diseases. They are also used for the prevention of post-harvest rots and as soil-drench treatments [9,19].

Hydrazone is another considerable pharmacophore group for antimicrobial activity. Some widely used antibacterial drugs such as furacilin, furazolidone and ftivazide are known to contain this group [20]. In past decades, hydrazones have received much attention and many studies [21–29] have been reported due to their chemotherapeutic value in the development of novel antimicrobial agents.

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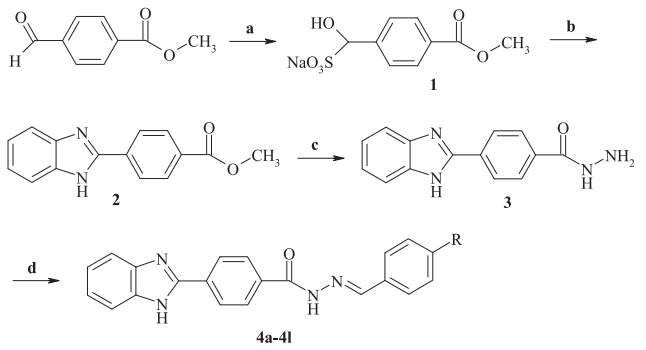
doi:10.1016/j.ejmech.2010.04.012

Looking at the antimicrobial importance of benzimidazole and hydrazone compounds it was thought that it would be worthwhile to design, synthesize some new benzimidazole derivatives bearing hydrazone moiety and screen them for potential antibacterial and antifungal activities. Furthermore, after extensive literature search, it was observed that, till date enough efforts have not been made to combine these two vital moieties as a single molecular scaffold and to study its antimicrobial activity. Consequently, prompted from the findings described above, we here reported synthesis and antimicrobial evaluation of some novel benzimidazole-hydrazone compounds.

2. Results and discussion

2.1. Chemistry

Present study was undertaken to synthesize some novel benzimidazole-hydrazone derivatives and investigate their probable antibacterial and antifungal effects. Target compounds were obtained at four steps. First of all, sodium disulfide adduct (**1**) of 4-formylbenzoic acid methyl ester was prepared in dilute EtOH. Secondly, *o*-phenylenediamine was reacted with the **1** in DMF to achieve 4-(1H-benzimidazole-2-yl)benzoic acid methyl ester (**2**) which was then treated with excess of hydrazine hydrate to obtain 4-(1H-benzimidazole-2-yl)benzoic acid hydrazide (**3**). At final step, reaction of the **3** with corresponding 4-substitutedbenzaldehyde derivative gave the title products (**4a**–**4l**). The structures of the obtained compounds were elucidated by spectral data. Significant stretching bands in the IR spectra were observed at expected regions. All of the aromatic and aliphatic protons in the 500 MHz ^{1}H NMR spectra were also recorded at estimated areas. The mass spectra (ES-MS) of the compounds showed [M + 1] peaks, in agreement with their molecular weight. Elemental analysis results for C, H and N elements were satisfactory within ±0.4% calculated values of the compounds. Synthesis procedure of the benzimidazole-hydrazone derivatives was outlined in Scheme 1. Some physicochemical properties and spectral data of the compounds were given in Table 1.



R: -H, -OH, -N(CH$_3$)$_2$, -Cl, -Br, -F, -CH$_3$, -OCH$_3$, -NO$_2$, -CF$_3$, COOH, CN

Reagents and conditions: **a:** $Na_2S_2O_5$, 80% EtOH, r.t 0.5h; **b:** o-phenylenediamine, DMF, 130 °C 4h; **c:** 80% $NH_2NH_2.H_2O$, EtOH, reflux 12h; **d:** corresponding 4-substitutedbenzaldehyde, catalytic amount CH_3COOH, n-ButOH, reflux 3h.

Scheme 1. Synthesis route of 4-substitutedbenzaldeyde *N*-[4-(1H-benzimidazol-2-yl) phenyl]hydrazone derivatives (**4a**–**4l**).

2.2. Microbiology

Final products were tested for their *in vitro* growth inhibitory activity against human pathogens. *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* and *Listeria monocytogenes* were evaluated as gram-positive bacteria. As gram-negative bacteria; *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 35218, *E. coli* ATCC 25922, *Salmonella typhimurium* NRRL B-4420, and *Proteus vulgaris* NRLL B-123 were tested. *Candida albicans*, *andida tropicalis* and *andida globrata* ATCC 36583 yeasts were also used in antimicrobial activity tests. Chloramphenicol and ketocanozole were used as control drugs. The observed antimicrobial data of the compounds and the reference drugs are given in Tables 2 and 3.

When compared with the reference drug chloramphenicol, most of the compounds in the series exhibited considerable antibacterial activity against *P. vulgaris* and *P. aeruginosa* (Table 2). The compounds **4b**, **4d**, **4e**, **4g** and the compounds **4a**–**4e**, **4h** were more potent than reference against *P. vulgaris* and *P. aeruginosa*, respectively. MIC value (50 μg/mL) of the other compounds in the series was equal to reference. Only the compound **4j** was found to be inactive against these two bacterial strains (Table 2).

The compounds **4d** and **4f**–**4h** showed significant antibacterial activity (MIC = 6.25 μg/mL) against *S. typhimurium*. Higher MIC value (25 μg/mL) than chloramphenicol against this bacterial strain was an introduction of antibacterial inability of the compounds **4b**, **4c** and **4j**. Antibacterial activity of the other five compounds was at same degree with the reference drug (Table 2).

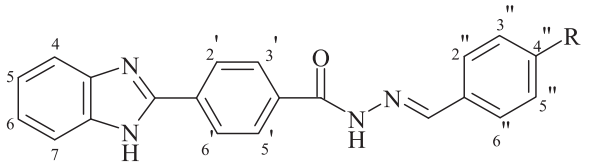
Some of the compounds exhibited equal antibacterial activity to chloramphenicol against *K. pneumoniae*, *S. aureus* and *E. faecalis*. Rest of them was found to be inactive against these bacterial strains. None of the compounds performed notable growth inhibitory activity against *E. coli* 35218, *E. coli* 25922, *L. monocytogenes* and *B. subtilis* bacterial strains (Tables 2 and 3).

Antifungal activity of the tested benzimidazole-hydrazone derivatives was observed as insignificant in comparison to their antibacterial activity. None of the synthesized compounds had greater activity then reference drug ketaconazole against any of the fungal strains. However, the compounds **4a**, **4d**, **4i** and the compounds **4d-f**, **4h**, **4j**, **4l** were found to be as active as ketaconazole against *C. albicans* and *C. tropicalis*, respectively (Table 3).

A chemical agent is valuable in medicinal field if only it possesses low toxicity with significant activity. Thus, toxicity of the compounds **4d**, **4e** and **4g** which have the highest antibacterial efficacy needs to be revealed. For this purpose *Brine-Shrimp* (*Artemia salina*) lethality assay was performed. This assay is regarded as a useful method for preliminary evaluation of toxicity, and it has been used for establishing of fungal toxins, plant extract toxicity, heavy metals, cyanobacteria toxins, pesticides, and cytotoxicity testing of dental materials [30], natural and synthetic organic compounds [31]. Moreover, *A. salina* 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 *A. salina* toxicity test, was slightly better than the rat tests for test compounds [32].

Toxicity test results were analyzed with the LC_{50} computer program (Trimmed Spearman–Karber Method, Version 1.5) so as to calculate LC_{50} values and 95% confidence intervals [33]. This program failed to give LC_{50} values of the compounds and 95% confidence intervals because number of dead larvae did not exceed 50% of total larvae. This was a significant result demonstrating that the tested compounds are non-toxic in the tested concentration range. Toxicity test results were presented in Table 4.

Table 1
Some physicochemical properties and spectral data of the compounds **4a**–**4l**.



No	R	M.P. (°C)	Yield (%)	IR (KBr, cm^{-1})	^1H NMR (500 MHz, DMSO-d_6)	ES-MS (M + 1, *m*/*z*)	Found (calculated)		
							C%	H%	N%
4a	−H	280	81	3284–3229 (N–H), 1655 (C=O), 1611–1463 (C=N and C=C).	7.10–7.36 (5H, m, H-5,6,3″,4″,5″), 7.50 (2H, m, H-4,7), 7.90–7.96 (4H, m, H-3′,5′,2″,6″), 8.14 (2H, d, J = 8.69 Hz, H-2′,6′), 8.46 (H, s, −N=CH−), 11.76 (H, br, N–H, *hydrazone*), 12.14 (H, br, N–H, *benzimidazole*).	341	74.10 (73.96)	4.74 (4.73)	16.46 (16.51)
4b	−OH	306	74	3281–3237 (N–H), 1661 (C=O), 1614–1460 (C=N and C=C).	7.10–7.21 (4H, m, H-5,6,3″,5″), 7.49 (2H, m, H-4,7), 7.91–7.96 (4H, m, H-3′,5′,2″,6″), 8.14 (2H, d, J = 8.61 Hz, H-2′,6′), 8.42 (H, s, −N=CH−), 9.93 (H, s, O–H), 11.71 (H, br, N–H, *hydrazone*), 12.08 (H, br, N–H, *benzimidazole*).	357	70.78 (70.85)	4.53 (4.55)	15.72 (15.69)
4c	$-N(CH_3)_2$	186	77	3287–3242 (N–H), 1659 (C=O), 1608–1459 (C=N and C=C).	2.98 (6H, s, $-N(CH_3)_2$), 6.91 (2H, d, J = 8.54, H-3″,5″), 7.10–7.20 (2H, m, H-5,6), 7.52 (2H, m, H-4,7), 7.90–7.96 (4H, m, H-3′,5′,2″,6″), 8.14 (2H, d, J = 8.60 Hz, H-2′,6′), 8.39 (H, s, −N=CH−), 11.68 (H, br, N–H, *hydrazone*), 12.08 (H, br, N–H, *benzimidazole*).	384	72.04 (71.84)	5.52 (5.51)	18.26 (18.20)
4d	−Cl	320	69	3293–3236 (N–H), 1648 (C=O), 1610–1461 (C=N and C=C).	7.10–7.20 (2H, m, H-5,6), 7.50–7.56 (4H, m, H-4,7, 3″,5″), 7.90–7.98 (4H, m, H-3′,5′,2″,6″), 8.14 (2H, d, J = 8.58 Hz, H-2′,6′), 8.51 (H, s, −N=CH−), 11.65 (H, br, N–H, *hydrazone*), 12.12 (H, br, N–H, *benzimidazole*).	376	67.29 (67.41)	4.03 (4.05)	14.95 (14.89)
4e	−Br	305	80	3271–3234 (N–H), 1657 (C=O), 1612–1464(C=N and C=C).	7.10–7.20 (2H, m, H-5,6), 7.50–7.65 (4H, m, H-4,7, 3″,5″), 7.90–8.02 (4H, m, H-3′,5′,2″,6″), 8.16 (2H, d, J = 8.49 Hz, H-2′,6′), 8.53 (H, s, −N=CH−), 11.63 (H, br, N–H, *hydrazone*), 12.11 (H, br, N–H, *benzimidazole*).	420	60.16 (60.10)	3.61 (3.62)	13.36 (13.49)
4f	-F	326	73	3286–3239 (N–H), 1658 (C=O), 1609–1460 (C=N and C=C).	7.10–7.20 (2H, m, H-5,6), 7.39–7.50 (4H, m, H-4,7, 3″,5″), 7.90–7.95 (4H, m, H-3′,5′,2″,6″), 8.16 (2H, d, J = 8.54 Hz, H-2′,6′), 8.49 (H, s, −N=CH−), 11.63 (H, br, N–H, *hydrazone*), 12.12 (H, br, N–H, *benzimidazole*).	357	70.38 (70.52)	4.22 (4.24)	15.63 (15.68)
4g	$-CH_3$	319	68	3290–3230 (N–H), 1659 (C=O), 1611–1462 (C=N and C=C).	2.34 (3H, s, $-CH_3$), 7.10–7.22 (4H, m, H-5,6,3″,5″), 7.50 (2H, m, H-4,7), 7.89–7.96 (4H, m, H-3′,5′,2″,6″), 8.14 (2H, d, J = 8.52 Hz, H-2′,6′), 8.44 (H, s, −N=CH−), 11.71 (H, br, N–H, *hydrazone*), 12.05 (H, br, N–H, *benzimidazole*).	355	74.56 (74.71)	5.12 (5.10)	15.81 (15.86)
4h	$-OCH_3$	289	74	3288–3241 (N–H), 1657 (C=O), 1610–1459 (C=N and C=C).	3.88 (3H, s, $-OCH_3$), 7.02 (2H, d, J = 8.47 Hz, H-3″,5″), 7.10–7.21 (2H, m, H-5,6,), 7.51 (2H, m, H-4,7), 7.89–7.95 (4H, m, H-3′,5′,2″,6″), 8.11 (2H, d, J = 8.53 Hz, H-2′,6′), 8.40 (H, s, −N=CH−), 11.64 (H, br, N–H, *hydrazone*), 12.08 (H, br, N–H, *benzimidazole*).	371	71.34 (71.29)	4.90 (4.90)	15.13 (15.17)
4i	$-NO_2$	335	73	3279–3235 (N–H), 1650 (C=O), 1612–1456 (C=N and C=C).	7.10–7.20 (2H, m, H-5,6,), 7.52 (2H, m, H-4,7), 7.92–8.09 (4H, m, H-3′,5′,2″,6″), 8.15 (2H, d, J = 8.56 Hz, H-2′,6′), 8.37 (2H, d, J = 8.13 Hz, H-3″,5″), 8.58 (H, s, −N=CH−), 11.67 (H, br, N–H, *hydrazone*), 12.09 (H, br, N–H, *benzimidazole*).	386	65.45 (65.57)	3.92 (3.90)	18.17 (18.11)

(*continued on next page*)

Table 1 (*continued*)

No	R	M.P. (°C)	Yield (%)	IR (KBr, cm^{-1})	^{1}H NMR (500 MHz, DMSO-d_6)	ES-MS (M + 1, *m/z*)	Found (calculated)		
							C%	H%	N%
4j	$-CF_3$	290	72	3285–3233 (N–H), 1652 (C=O), 1611–1463 (C=N and C=C).	7.10–7.21 (2H, m, H-5,6,), 7.52 (2H, m, H-4,7), 7.92–8.06 (4H, m, H-3′,5′,2″,6″), 8.14 (2H, d, *J* = 8.48 Hz, H-2′,6′), 8.27 (2H, d, *J* = 8.19 Hz, H-3″,5″), 8.55 (H, s, –N=CH–), 11.64 (H, br, N–H, *hydrazone*), 12.07 (H, br, N–H, *benzimidazole*).	409	64.70 (64.81)	3.70 (3.68)	13.72 (13.69)
4k	–COOH	345	80	3295–3244 (N–H), 1656 (C=O), 1608–1461 (C=N and C=C).	7.10–7.19 (2H, m, H-5,6,), 7.52 (2H, m, H-4,7), 7.92–8.05 (4H, m, H-3′,5′,2″,6″), 8.14 (2H, d, *J* = 8.48 Hz, H-2′,6′), 8.25 (2H, d, *J* = 8.24 Hz, H-3″,5″), 8.52 (H, s, –N=CH–), 11.64 (H, br, N–H, *hydrazone*), 12.12 (2H, br, –COOH and N–H, *benzimidazole*).	385	68.74 (68.97)	4.20 (4.19)	14.58 (14.53)
4l	–CN	322	76	3286–3231 (N–H), 1654 (C=O), 1613–1458 (C=N and C=C).	7.10–7.20 (2H, m, H-5,6,), 7.52 (2H, m, H-4,7), 7.91–8.07 (4H, m, H-3′,5′,2″,6″), 8.15 (2H, d, *J* = 8.56 Hz, H-2′,6′), 8.29 (2H, d, *J* = 8.11 Hz, H-3″,5″), 8.54 (H, s, –N=CH–), 11.66 (H, br, N–H, *hydrazone*), 12.09 (H, br, N–H, *benzimidazole*).	366	72.32 (72.11)	4.14 (4.16)	19.17 (19.10)

Table 2
MIC values (μg/mL) of benzimidazole-hydrazone derivatives against gram-negative bacterial strains.

Compound	A	B	C	D	E	F
4a	25	100	50[a]	12.5[a]	25	25[b]
4b	25	100	25[b]	25	25	25[b]
4c	25	100	50[a]	25	50	25[b]
4d	25	100	25[b]	6.25[b]	12.5[a]	25[b]
4e	25	50	25[b]	12.5[a]	12.5[a]	25[b]
4f	25	50	50[a]	6.25[b]	25	50[a]
4g	25	100	25[b]	6.25[b]	12.5[a]	50[a]
4h	25	50	50[a]	6.25[b]	25	25[b]
4i	25	100	50[a]	12.5[a]	25	50[a]
4j	25	100	50[a]	12.5[a]	25	50[a]
4k	50	200	100	25	50	100
4l	25	100	50[a]	12.5[a]	12.5[a]	50[a]
Ref.	12.5	12.5	50	12.5	12.5	50

A: *Escherichia coli* 35218; B: *Escherichia coli* 25922; C: *Proteus vulgaris*; D: *Salmonella thyphimurium*; E: *Klebsiella pneumoniae*; F: *Pseudomonas aeruginosa.*
Reference: Chloramphenicol.
[a] Equal MIC value to reference.
[b] Lower MIC value than reference.

2.3. SAR studies

The substitution pattern of the benzimidazole-hydrazone derivatives was carefully selected to confer different electronic environment to the molecules. Thus, electron donating groups to aromatic ring, such as halogens, methyl, methoxy, hydroxyl and dimethylamine and electron withdrawing groups from aromatic ring, such as nitro, trifluoromethyl, carboxyl and cyano were chosen as substituents on the chemical structure of the target compounds. The compounds **4i**–**4l**, bearing electron withdrawing groups, did not exhibit stronger antibacterial activity than reference drug chloramphenicol against any of the bacterial strains. However, nitro, trifluoromethyl and cyano substituted compounds **4i**, **4j** and **4l** found to be as effective as reference drug against some of the bacterial strains (Tables 2 and 3). On the other hand, the compounds **4b**–**4h** which were prepared to contain electron donating groups, showed lower MIC value then chloramphenicol against at least one bacterial strain. Among the compounds, chloro, bromo and methyl substituted ones **4d**, **4e** and **4g** possessed wider antibacterial spectrum than the others (Tables 2 and 3). The compound **4d** was the most active derivative in the series, because MIC value of it was not only lower than the reference drug against *P. vulgaris*, *S. thyphimurium* and *P. aeruginosa* but also equal to

Table 3
MIC values (μg/mL) of benzimidazole-hydrazone derivatives against gram-positive bacterial and fungal strains.

Compound	A	B	C	D	E	F	G
4a	400	25	12.5[a]	25	50[a]	100	100
4b	400	50	25	25	100	100	100
4c	200	50	25	25	100	50	100
4d	100	12.5[a]	12.5[a]	25	50[a]	50	50[a]
4e	200	25	12.5[a]	25	100	100	50[a]
4f	200	50	25	25	100	100	50[a]
4g	200	25	12.5[a]	50	100	100	100
4h	200	25	12.5[a]	25	100	50	50[a]
4i	200	25	25	50	50[a]	100	100
4j	200	25	12.5[a]	50	100	100	50[a]
4k	400	50	50	50	200	200	200
4l	200	25	12.5[a]	50	100	100	50[a]
Ref. 1	50	12.5	12.5	12.5	–	–	–
Ref. 2	–	–	–	–	50	25	50

A: *Listeria monocytogenes*; B: *Staphylococcus aureus*; C: *Enterococcus faecalis*; D: *Bacillus subtilis*; E: *Candida albicans*; F: *Candida globrata*; G: *Candida tropicalis.*
Reference 1: Chloramphenicol: Reference 2: Ketaconazole.
[a] Equal MIC value to reference.

Table 4
Brine-shrimp toxicity results of the compounds **4d**, **4e,** and **4g**.

Concentration (μg/mL)	Mortality[a]		
	4d	**4e**	**4g**
1000	3	4	3
500	3	3	3
250	3	3	2
125	1	2	1
62.5	1	2	1
31.25	1	1	1
15.625	1	1	1
7.8125	1	1	1
3.906	1	1	1
Control	1	1	1
LC50	>1000 μg/mL	>1000 μg/mL	>1000 μg/mL
Toxicity	Non-toxic	Non-toxic	Non-toxic

[a] Ten organisms (*Artemia salina*) tested for each concentration.

reference against *K. pneumoniae*, *S. aureus* and *E.s faecalis*. Besides, MIC values of the other derivatives were not lower than the **4d** against any of these bacterial strains (Tables 2 and 3).

In order to clarify relationship between antibacterial activity and electronic properties of the target compounds some electronic parameters of the substituents were evaluated. For this purpose, electronic substituent constant (σ), field constant (F), resonance effect constant (R) and polarizabilitiy (α) of the compounds were determined (Table 5). Number of the bacterial strains against which the test compounds exhibited valuable antibacterial effect (lower or equal MIC value when compared with reference) were counted and then probable relationship between number of effected bacterial strains and electronic substituent constants were sought.

After examining the Table 5, no relationship between antibacterial activity and σ, F or α constants was established. On the other hand, a relationship between R values and number of effected bacterial strains was determined. Order of this constant from the most electron withdrawing (−CN and $-CF_3$, $R = 0.19$) substituent to most electron donating ($-N(CH_3)_2$, $R = -0.92$) one and number of effected bacterial strains were concordant at a point. As seen in Table 5, it is very clear that electron donating abilities of the chloro ($R = -0.15$), bromo ($R = -0.17$) and methyl ($R = -0.13$) groups, which are the substituents of the most active compounds **4d**, **4e** and **4g**, are very close. Besides, the number of significantly inhibited strains by the **4d**, **4e** and **4g** is higher than the other compounds (Table 5). This finding indicates that according to chloro, bromo and methyl substituents, increasing electron withdrawing or donating ability causes to decrease on the number of effected strains. Hence, it can be declared that chloro, bromo and methyl substituents bearing derivatives are the most suitable compounds for achieving the best antibacterial spectrum. The reason for this result may be explained by electron density of the compounds. It has been reported that electron-donating groups increase the electron density which makes the compounds effective against microorganisms and enhances the antibacterial activity [28]. However, high electron density causes more difficult diffusion through the bacteria cell and substantial activity loss may occur [34]. Thus, for a compound an optimum electron density is inevitable so as to gain a significant antibacterial activity. As a result it can be thought that electron donating ability of the chloro, bromo, and methyl groups contributes to the compounds **4d**, **4e** and **4g** to reach an optimum electron density which is important for antibacterial activity.

Table 5
Some electronic constants of compounds **4a**–**4l** and number of inhibited bacterial strains.

Compound	R	σ[a]	F[a]	R[a]	α[b]	NBS[c]
4a	−H	0.00	0.00	0.00	39.95	4
4b	−OH	−0.37	0.29	−0.64	40.53	2
4c	$-N(CH_3)_2$	−0.83	0.10	−0.92	44.76	2
4d	−Cl	0.23	0.41	−0.15	41.83	6
4e	−Br	0.23	0.44	−0.17	42.54	5
4f	−F	0.06	0.43	−0.34	39.54	3
4g	$-CH_3$	−0.17	−0.04	−0.13	41.72	5
4h	$-OCH_3$	−0.27	0.26	−0.51	42.45	4
4i	$-NO_2$	0.78	0.67	0.16	41.74	3
4j	$-CF_3$	0.54	0.38	0.19	40.81	4
4k	−COOH	0.45	0.33	0.15	42.22	0
4l	−CN	0.66	0.51	0.19	41.76	4

[a] Electronic substituent constant taken from Hansch et al. [37].
[b] Calculated using ChemAxon/MarvinSketch computer program.
[c] Number of bacterial strains against which the test compounds exhibited lower or equal MIC value when compared with reference.

3. Conclusion

The preliminary *in vitro* antibacterial, antifungal and toxicological screening results of novel benzimidazole-hydrazone derivatives reported here have indicated the antimicrobial potent of the synthesized compounds. Most effective compounds were found to be non-toxic in *A. salina* toxicity test. Performed SAR observation has showed the importance of electronic environment on antimicrobial activity. The presence of chloro, bromo and methyl substituents on the aromatic ring has increased the activity of the compounds compared to those with other substituents. Findings from the SAR and toxicity studies have encouraged us to make some modifications on basic structure of the obtained compounds to achieve selective, more active and non-toxic derivatives in ongoing studies. In addition, for further investigations these findings can have a good effect on medicinal chemists to synthesize similar compounds selectively bearing chloro, bromo and methyl substituents.

4. Materials and methods

4.1. Chemistry

All of the chemicals used in syntheses were obtained from Merck. Melting points (m.p) of target compounds were measured in Electrothermal 9001 Digital Melting Point Apparatus and uncorrected. IR spectra were recorded on Shimadzu, 8400 FTIR spectrometer as KBr pellets. ^{1}H NMR spectra were recorded on Bruker UltraShield 500 MHz spectrometer in deutoro dimethyl sulfoxide. ES-MS data were obtained on Agilent 1100 Series LC/MSD Trap VL&SL spectrometer. Elemental analyses (C, H, and N) were determined on a Perkin Elmer analyser.

4.1.1. 4-(1H-benzimidazole-2-yl)benzoic acid methyl ester (**2**)

4-Formylbenzoic acid methyl ester (16.4 g, 100 mmol) and sodium disulfide (9.5 g, 50 mmol) were dissolved in 100 mL of aqueous EtOH (80%) and stirred at room temparature for 30 min. 20 mL of EtOH was added and allowed to cool in an ice bath for a several hours. The precipitate was filtered and dried (yield% 91). The mixture of this salt (21.44 g, 80 mmol) and *o*-phenylenediamine (8.64 g, 80 mmol) in DMF (40 mL) were heated at 130 °C for 4 h. The reaction mixture was poured into ice-water and the solid was filtered, crystallisation of crude product from EtOH gave the **2**. Yield: 63%. m.p. 228 °C. IR (KBr, ν_{maks} cm^{-1}): 1463–1608 (C=C and C=N), 1737 (C=O), 3284–3231 (N–H).

4.1.2. 4-(1H-benzimidazole-2-yl)benzoic acid hydrazide (**3**)

To a suspansion of the **2** (10.04 g, 40 mmol) in 50 mL EtOH, 15 mL hydrazine hydrate (80%) was added. Reaction mixture was

refluxed for 24 h. Then the solvent and excess of hydrazine hydrate was evaporated and the residue was washed with water, filtered, dried, and then crystallized from EtOH. Yield % 78. m.p. 304 °C. IR (KBr, ν_{maks} cm^{-1}): 1458–1604 (C=C and C=N), 1658 (C=O), 3277–3234 (N–H).

4.2. General synthesis procedure of 4-Substitutedbenzaldeyde N-[4-(1H-benzimidazol-2-yl)phenyl] hydrazone derivatives (**4a**–**4l**)

Equimolar quantities (2 mmol) of **3** and appropriate 4-substitutedbenzaldehydes in 25 mL of butanol were refluxed for 3 h with the presence of catalytic amount of glacial acetic acid. The resulting solid was filtered and recrystallized from EtOH. Some physicochemical properties and spectral data of the compounds were given in Table 1.

4.3. Microbiology

The study was designed to compare MICs obtained by the CLSI reference M7–A7 broth microdilution method [35,36]. MIC readings were performed twice for each chemical agent. For both the antibacterial and antifungal assays the compounds were dissolved in DMSO. Further dilutions of the compounds 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.

4.3.1. Antimicrobial assay

The cultures were obtained from Mueller–Hinton broth (Difco) for the bacterial strains after overnight incubation at 35 ± 1 °C. The yeasts were maintained in Sabouroud dextrose broth (Difco) after overnight incubation 35 ± 1 °C. The inocula of test microorganisms adjusted to match the turbidity of a Mac Farland 0.5 standard tube as determined with a spectrophotometer and the final inoculum size was $0.5–2.5 \times 10^5$ cfu/mL for antibacterial and antifungal assays. Testing was carried out in Mueller–Hinton broth and Sabouroud dextrose broth (Difco) at pH 7 and the two-fold serial dilutions technique was applied. The last well on the microplates containing only inoculated broth was kept as controls and the last well with no growth of microorganism was recorded to represent the MIC expressed in μg/mL. Each experiment in the antimicrobial assays was replicated twice in order to define the MIC values. Chloramphenicol and ketocanozole were used as control drugs.

4.3.2. Brine-shrimp lethality assay

Brine-shrimp toxicity assay was used to determine cytotoxicity levels of the most active compounds (**4d**, **4e**, **4g**). 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 (1000–3.906 μg/ml). In order to prevent the toxicity results from possible false effect originated from DMSO's toxicity, stock solutions of the compounds 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 [31]. Pure DMSO was used as a positive control for the toxicity assay. Fresh eggs of *A. salina* were purchased from the local pet shop, Eskisehir/Turkey. The eggs were hatched in a conical flask containing 300 ml artificial seawater made by dissolving a commercial marine salt in deionised 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 were hatched within 48 h. Ten larvae were transferred with pipetter into each vial containing test compound and artificial seawater. 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.

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