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Antimicrobial Activity of a New Series of Benzimidazole Derivatives

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Due to antimicrobial importance of benzimidazoles and hydrazones, some benzimidazole-hydrazone compounds were synthesized to screen their antimicrobial activity. Structures of the synthesized compounds were elucidated by ^{1}H-NMR, IR and ES-MS spectral data and elemental analysis. The synthesized benzimidazole-hydrazones exhibited very weak antibacterial activity. However, antifungal activity of some of the synthesized compounds was very notable against *Candida* species. The compounds displaying important antifungal activity were screened for their toxicity. *Artemia salina* 96-well assay was used to determine cytotoxicity of the compounds. Tested compounds exhibited toxicity to different extents (LD_{50} = 126.33-368.72 μg/mL). Nevertheless, determination of 3-14 folds higher LD_{50} than minimum inhibitory concentration is a significant finding, which demonstrates that the compounds display antifungal activity at non-toxic concentration.

Key words: Benzimidazole, Hydrazone, Antibacterial, Antifungal, *Artemia salina*

INTRODUCTION

In the past 25 years, the incidence of microbial infections has increased on alarming levels over the world as a result of antimicrobial resistance (Bayrak et al., 2009) and possible microbial implications for morbidity, mortality and health care costs have become a serious fear (Cosgrove, 2006). In order to overcome these challenges, new agents should preferably consist of chemical characteristics that clearly differ from those of existing agents. Thus, there is a pressing need for development of novel antimicrobial agents. In drug design and development field, a critical part of the search for novel drugs is the synthesis of compounds that are new yet resemble known biologically active molecules by virtue of the presence of essential structural features (Silverman, 1992; Cunha, 1998; Levy, 1998; Lipsitch, 2001).

Benzimidazoles fit above requirement well since they have shown a chemotherapeutic importance. Several thousands of benzimidazole analogs have been synthesized and screened for their antimicrobial activity up-to-date (Podunavac-Kuzmanovic et al., 2008). The cause of a special notice on benzimidazole compounds has been cyanocobalamine (vitamin B_{12}) which bears 5,6-dimethylbenzimidazole fragment and is capable of inducing the growth of bacteria. Nevertheless, the benzimidazole shows structural similarity to purine and hence; antibacterial capability of benzimidazoles is explained by their competition with purines resulting in inhibition of the synthesis of bacterial nucleic acids and proteins (Spasov et al., 1999; Arjmand et al., 2005). In addition to their antibacterial activity, benzimidazoles can be classified as one of the most important groups 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 are some main examples of this fungicide class (Kaplancikli et al., 2004; Ozdemir et al., 2010). In this group, the well-known fungicide thiabendazole inhibits fungal microtubular function, resulting in non-disjunction of chromosomes at cell division (Allen and Gootlied, 1970; Watanabe-Akanuma et al., 2005).

As well as benzimidazoles, hydrazide-hydrazones

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are the other pharmacophores which have been claimed to exhibit appreciable antibacterial and antifungal activity. Furacilin, furazolidone, ftivazide and nifuroxazide are the well-known antimicrobial agents, which contain hydrazide-hydrazones moieties (Chornous et al., 2001). Due to their chemotherapeutic potency, attention through the hydrazide-hydrazones has increased and lots of studies related with their antimicrobial activity have been reported in recent years (Papakonstantinou-Garoufalias et al., 2002; Rollas et al., 2002; Vicini et al., 2002; Loncle et al., 2004; Masunari and Tavares, 2007; Salgin-Goksen et al., 2007; Joshi et al., 2008; Mostafa et al., 2008; Kumar et al., 2009; Ozdemir et al., 2009).

In the light of above observations, we have recently performed a study (Ozkay et al., 2010) including the synthesis of some benzimidazole-hydrazone derivatives and evaluation of a relationship between their structures and antimicrobial activity. Results of such investigation have encouraged us to make some structural modifications to improve biological activity. Hence, we have designed some synthesis strategies. One of these strategies includes the synthesis of a compact system bearing 2-aminobenzimidazole and hydrazone moieties. The reason for this choice can be explained by the pharmacological importance of 2-aminobenzimidazole, which has approximately 30 derivatives registered in the world as drugs exhibiting diverse pharmacological activities such as antiparasitic, antifungal, and antiviral (Nawrocka et al., 2004).

As a result, in connection with our recent study, we synthesized a new class of compounds bearing 2-aminobenzimidazole and hydrazone moieties in order to develop new antimicrobial agents.

MATERIALS AND METHODS

Chemistry

All reagents were used as purchased from commercial suppliers (Merck, Acros or Sigma-Aldrich) without further purification. Melting points (M.p.) were determined by using an Electrothermal 9100 digital melting point apparatus and were uncorrected. During the synthesis, the compounds were routinely checked for purity by TLC on 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 analyser.

Synthesis of the starting materials (1 and 2)

Methyl 4-(1H-benzimidazol-2-yl-iminomethyl) benzoate (1) and its reduction product methyl 4-(1H-benzimidazol-2-yl-aminomethyl) benzoate (2) were synthesized in accordance with the previous method (Nawrocka et al., 2004), in which reaction of 2-aminobenzimidazole with various benzaldehyde derivatives and reduction of the obtained products by $NaBH_4$ were described.

4-(1H-Benzimidazol-2-yl-aminomethyl) benzohydrazide (3)

Methyl 4-(1H-benzimidazol-2-yl-aminomethyl) benzoate (**2**) (10 g, 35.58 mmol) was dissolved in 50 mL of EtOH and 15 mL of 80% hydrazine hydrate was added. Reaction mixture was refluxed for 12 h. Solvent and excess of hydrazine hydrate was evaporated and the residue was washed with water, filtered, dried, and then crystallized from EtOH. Yield 73%; M. p. 220-222 °C; IR [ν, cm^{-1}, KBr]: 3416-3279 (N-H), 1678 (C=O), 1603-1457 (C=C and C=N).

General synthesis procedure for 4-(1H-benzimidazol-2-yl-aminomethyl)-N'-(4-substituted benzylidene) benzohydrazides (4a-4n)

Equimolar quantities of 4-(1H-benzimidazol-2-yl-aminomethyl) benzohydrazide (**3**) (0.562 g, 2 mmol) and appropriate 4-substituted benzaldehyde (2 mmol) in 25 mL of butanol were refluxed for 3 h with the presence of catalytic amount of glacial AcOH. Reaction mixture was allowed to cool down and then resulting solid was filtered and recrystallized from EtOH to give the title products (**4a**-**4n**).

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-(benzylidene) benzohydrazide (4a)

Yield 67%; M. p. 157-158°C; IR [ν, cm^{-1}, KBr]: 3394-3285 (N-H), 1684 (C=O), 1610-1453 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.39 (2H, d, J = 4.52 Hz, NH-$\underline{CH_2}$), 7.06-7.52 (10H, m, $\underline{NH}$-CH_2 and Ar-H), 7.82-7.94 (4H, m, Ar-H), 8.39 (1H, s, N=CH), 11.73 (1H, s, CO-NH), 12.33 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 370.5 [M+1, 100%]. Anal. calcd. for $C_{22}H_{19}N_5O$: C, 71.53; H, 5.18; N, 18.96. Found: C, 71.69; H, 5.15; N, 18.88.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(dimethylamino)benzylidene] benzohydrazide (4b)

Yield 73%; M. p. 190°C (dec.); IR [ν, cm^{-1}, KBr]: 3383-3281 (N-H), 1677 (C=O), 1607-1451 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 13.02 (6H, s, $N(CH_3)_2$), 4.37 (2H, d, J = 4.59 Hz, NH-$\underline{CH_2}$), 6.84-7.51 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.79-7.91 (4H, m, Ar-H), 8.34 (1H, s, N=CH), 11.71 (1H, s, CO-NH), 12.39 (1H,

br, N-H, *benzimidazole*); ES-MS (*m/z*): 413.7 [M+1, 100%]. Anal. calcd. for $C_{24}H_{24}N_6O$: C, 69.88; H, 5.86; N, 20.37. Found: C, 69.78; H, 5.88; N, 20.31.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(diethylamino)benzylidene] benzohydrazide (4c)
Yield 64%; M. p. 262-263ºC; IR [ν, cm^{-1}, KBr]: 3391-3287 (N-H), 1682 (C=O), 1608-1456 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 1.12 (6H, t, J = 7.04 Hz, N(CH_2-$\underline{CH_3}$)$_2$), 3.43 (4H, q, J = 7.11 Hz, N($\underline{CH_2}$-CH_3)$_2$), 4.39 (2H, d, J = 4.46 Hz, NH-$\underline{CH_2}$), 6.77-7.51 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.81-7.93 (4H, m, Ar-H), 8.38 (1H, s, N=CH), 11.74 (1H, s, CO-NH), 12.36 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 441.6 [M+1, 100%]. Anal. calcd. for $C_{26}H_{28}N_6O$: C, 70.89; H, 6.41; N, 19.08. Found: C, 70.37; H, 6.44; N, 19.06.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(hydroxy)benzylidene] benzohydrazide (4d)
Yield 76%; M. p. 243ºC (dec.); IR [ν, cm^{-1}, KBr]: 3528 (O-H), 3402-3278 (N-H), 1686 (C=O), 1605-1457 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.40 (2H, d, J = 4.57 Hz, NH-$\underline{CH_2}$), 6.94-7.54 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.83-7.93 (4H, m, Ar-H), 8.39 (1H, s, N=CH), 9.74 (1H, s, O-H), 11.73 (1H, s, CO-NH), 12.38 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 386.6 [M+1, 100%]. Anal. calcd. for $C_{22}H_{19}N_5O_2$: C, 68.56; H, 4.97; N, 18.17. Found: C, 68.86; H, 4.97; N, 18.21.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(methyl)benzylidene] benzohydrazide (4e)
Yield 74%; M. p. 226ºC; IR [ν, cm^{-1}, KBr]: 3384-3279 (N-H), 1679 (C=O), 1601-1460 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 2.29 (3H, s, CH_3), 4.37 (2H, d, J = 4.63 Hz, NH-$\underline{CH_2}$), 7.04-7.52 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.83-7.91 (4H, m, Ar-H), 8.38 (1H, s, N=CH), 11.71 (1H, s, CO-NH), 12.36 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 384.5 [M+1, 100%]. Anal. calcd. for $C_{23}H_{21}N_5O$: C, 69.16; H, 5.30; N, 17.53. Found: C, 69.40; H, 5.29; N, 17.45.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(methoxy)benzylidene] benzohydrazide (4f)
Yield 59%; M. p. 310-311ºC; IR [ν, cm^{-1}, KBr]: 3389-3282 (N-H), 1681 (C=O), 1612-1455 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 3.85 (3H, s, OCH_3), 4.36 (2H, d, J = 4.55 Hz, NH-$\underline{CH_2}$), 6.99-7.51 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.84-7.93 (4H, m, Ar-H), 8.34 (1H, s, N=CH), 11.73 (1H, s, CO-NH), 12.42 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 400.4 [M+1, 100%]. Anal. calcd. for $C_{23}H_{21}N_5O_2$: C, 72.04; H, 5.52; N, 18.26. Found: C, 72.16; H, 5.53; N, 18.29.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(ethoxy)benzylidene] benzohydrazide (4g)
Yield 63%; M. p. 231ºC; IR [ν, cm^{-1}, KBr]: 3401-3296 (N-H), 1683 (C=O), 1607-1453 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 1.17 (3H, t, J = 7.22 Hz, $OCH_2\underline{CH_3}$), 4.02 (2H, q, J = 7.26 Hz, $O\underline{CH_2}CH_3$), 4.37 (2H, d, J = 4.38 Hz, NH-$\underline{CH_2}$), 7.01-7.52 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.82-7.92 (4H, m, Ar-H), 8.35 (1H, s, N=CH), 11.73 (1H, s, CO-NH), 12.34 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 414.5 [M+1, 100%]. Anal. calcd. for $C_{24}H_{23}N_5O_2$: C, 69.72; H, 5.61; N, 16.94. Found: C, 69.46; H, 5.63; N, 16.96.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(chloro)benzylidene] benzohydrazide (4h)
Yield 69%; M. p. 214-215ºC; IR [ν, cm^{-1}, KBr]: 3397-3278 (N-H), 1687 (C=O), 1608-1453 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.37 (2H, d, J = 4.64 Hz, NH-$\underline{CH_2}$), 7.04-7.51 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.79-7.91 (4H, m, Ar-H), 8.33 (1H, s, N=CH), 11.75 (1H, s, CO-NH), 12.32 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 404.9 [M+1, 100%], 405.9 [M+2, 31%], 406.9 [M+3, 14%]. Anal. calcd. for $C_{22}H_{18}ClN_5O$: C, 65.43; H, 4.49; N, 17.34. Found: C, 65.22; H, 4.50; N, 17.33.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(bromo)benzylidene] benzohydrazide (4i)
Yield 71%; M. p. 202ºC; IR [ν, cm^{-1}, KBr]: 3409-3286 (N-H), 1684 (C=O), 1605-1452 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.35 (2H, d, J = 4.41 Hz, NH-$\underline{CH_2}$), 7.03-7.50 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.81-7.93 (4H, m, Ar-H), 8.34 (1H, s, N=CH), 11.76 (1H, s, CO-NH), 12.38 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 448.5 [M+1, 100%], 449.5 [M+2, 28%], 450.6 [M+3, 12%]. Anal. calcd. for $C_{22}H_{18}BrN_5O$: C, 58.94; H, 4.05; N, 15.62. Found: C, 59.11; H, 4.04; N, 15.67.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(fluoro)benzylidene] benzohydrazide (4j)
Yield 63%; M. p. 221-223ºC; IR [ν, cm^{-1}, KBr]: 3394-3281 (N-H), 1676 (C=O), 1609-1455 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.37 (2H, d, J = 4.57 Hz, NH-$\underline{CH_2}$), 7.05-7.51 (9H, m, $\underline{NH}$-CH_2 and Ar-H), 7.84-7.93 (4H, m, Ar-H), 8.36 (1H, s, N=CH), 11.72 (1H, s, CO-NH), 12.40 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 388.5 [M+1, 100%]. Anal. calcd. for $C_{22}H_{18}FN_5O$: C, 68.21; H, 4.68; N, 18.08. Found: C, 68.04; H, 4.69; N, 18.13.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(nitro)benzylidene] benzohydrazide (4k)
Yield 82%; M. p. 254-256ºC; IR [ν, cm^{-1}, KBr]: 3396-

3282 (N-H), 1683 (C=O), 1613-1458 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.36 (2H, d, J = 4.38 Hz, NH-$\underline{CH_2}$), 7.09-7.51 (7H, m, $\underline{NH}$-CH_2 and Ar-H), 7.91-8.06 (4H, m, Ar-H), 8.33-8.37 (3H, m, N=CH and Ar-H), 11.74 (1H, s, CO-NH), 12.37 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 415.6 [M+1, 100%]. Anal. calcd. for $C_{22}H_{18}N_6O_3$: C, 63.76; H, 4.38; N, 20.28. Found: C, 63.85; H, 4.36; N, 20.23.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(trifluoro)benzylidene] benzohydrazide (4l)

Yield 78%; M. p. 304°C; IR [ν, cm^{-1}, KBr]: 3398-3285 (N-H), 1691 (C=O), 1602-1451 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.38 (2H, d, J = 4.43 Hz, NH-$\underline{CH_2}$), 7.08-7.53 (7H, m, $\underline{NH}$-CH_2 and Ar-H), 7.91-8.02 (4H, m, Ar-H), 8.23 (2H, d, J = 8.23 Hz, Ar-H), 8.34 (1H, s, -N=CH-), 11.73 (1H, s, CO-NH), 12.43 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 438.6 [M+1, 100%]. Anal. calcd. for $C_{23}H_{18}F_3N_5O$: C, 63.15; H, 4.15; N, 16.01. Found: C, 62.96; H, 4.17; N, 15.96.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(cyano)benzylidene] benzohydrazide (4m)

Yield 81%; M. p. 198°C; IR [ν, cm^{-1}, KBr]: 3404-3283 (N-H), 1677 (C=O), 1606-1454 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.37 (2H, d, J = 4.42 Hz, NH-$\underline{CH_2}$), 7.08-7.51 (7H, m, $\underline{NH}$-CH_2 and Ar-H), 7.92-8.05 (4H, m, Ar-H), 8.28 (2H, d, J = 8.56 Hz, Ar-H), 8.34 (1H, s, -N=CH-), 11.73 (1H, s, CO-NH), 12.39 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 395.5 [M+1, 100%]. Anal. calcd. for $C_{23}H_{18}N_6O$: C, 70.04; H, 4.60; N, 21.31. Found: C, 69.83; H, 4.59; N, 21.33.

4-(1H-Benzimidazol-2-yl-aminomethyl)-N'-[4-(carboxy)benzylidene] benzohydrazide (4n)

Yield 77%; M. p. 311°C; IR [ν, cm^{-1}, KBr]: 3468 (O-H), 3399-3287 (N-H), 1681 (C=O), 1609-1451 (C=N and C=C); ^{1}H-NMR (500 MHz, DMSO-d_6, δ ppm): 4.36 (2H, d, J = 4.54 Hz, NH-$\underline{CH_2}$), 7.08-7.52 (7H, m, $\underline{NH}$-CH_2 and Ar-H), 7.91-8.04 (4H, m, Ar-H), 8.19 (2H, d, J = 8.53 Hz, Ar-H), 8.35 (1H, s, -N=CH-), 11.71 (1H, s, CO-NH), 12.40 (1H, br, N-H, *benzimidazole*); ES-MS (*m/z*): 414.5 [M+1, 100%]. Anal. calcd. for $C_{23}H_{19}N_5O_3$ (413.44): C, 66.82; H, 4.63; N, 16.94. Found: C, 66.87; H, 4.64; N, 16.88.

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; *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 35218, *E. coli* ATCC 25922, *Salmonella thyphimurium* NRRL B-4420 and *Proteus vulgaris* NRLL B-123 and yeast as *Candida albicans*, *Candida tropicalis* and *Candida globrata* ATCC 36583 (obtained from Faculty of Medicine Osmangazi University, Eskisehir, Turkey). Chloramphenicol and ketoconazole were used as control drugs.

Some of the synthesized compounds 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.

Antimicrobial activity assay

Antimicrobial activity test was performed according to CLSI reference M7-A7 and M100-S16 broth microdilution methods as described in our previous study (Ozkay et al., 2010). Twice minimum inhibitory concentration (MIC) readings were carried out for each chemical agent. The compounds were dissolved in DMSO for antibacterial and antimycotic assays. 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.

Brine-Shrimp lethality assay

Artemia salina 96-well assay has been used to determine the cytotoxicity levels of the compounds. Tested compounds were prepared within the range of 1.95-1000 μg/mL, by dissolving in DMSO. The LD_{50} values and 95% confidence intervals of the compounds were calculated with the LC_{50} computer program (Trimmed Spearman-Karber Method, Version 1.5) (Calleja and Persoone, 1992; Choudhary and Thomsen, 2001). The experimental procedure details were explained in our recent study (Ozkay et al., 2010).

RESULTS AND DISCUSSION

Chemistry

In the present work, 14 novel benzimidazole-hydrazone compounds were synthesized. In the first step, methyl 4-(1H-benzimidazol-2-yl-iminomethyl) benzoate (**1**) was prepared by reacting 2-aminobenzimidazole

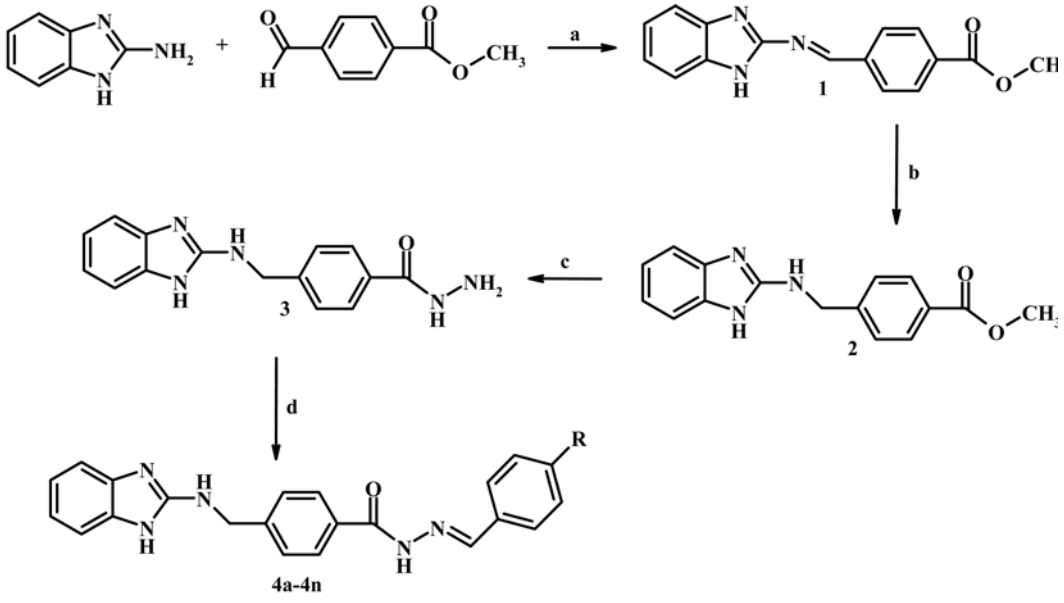
with methyl 4-formylbenzoate in the mixture of absolute ethanol/benzene (5:1), a few drops of glacial acetic acid used as a catalyst. Secondly, reduction of 1 with a chemoselective reducing agent $NaBH_4$ in isopropanol gave the methyl 4-(1H-benzimidazol-2-yl-aminomethyl) benzoate (**2**). In the third reaction step, compound **2** was treated with excess of hydrazine hydrate (80%) in absolute ethanol to obtain 4-(1H-benzimidazol-2-yl-aminomethyl) benzohydrazide (**3**). Finally, reaction of **3** and corresponding benzaldehyde derivatives in butanol with the presence of catalytic amounts of glacial acetic acid gave the 4-(1H-benzimidazol-2-yl-aminomethyl)-N'-(4-substituted benzylidene) benzohydrazides (**4a**-**4n**). Synthetic pathway of the compounds is given in Scheme 1.

Formulas of the compounds (**4a**-**4n**) were confirmed by elemental analyses and their structures were elucidated by IR, ^{1}H-NMR and ES-MS spectral data. In the IR spectra N-H, C=O, C=N and C=C functions absorbed strongly in the expected regions: N-H at 3404-3278 cm^{-1}, C=O at 1691-1676 cm^{-1}, C=N and C=C at 1613-1451 cm^{-1}, respectively. The ^{1}H-NMR spectra showed methylene protons of methylamine (NH-CH_2) at 4.35-4.40 ppm, as doublet, in all of the compounds. In the compounds **4a**-**4j**, the amine protons of methylamine and the aromatic protons belonging to benzimidazole, 3th and 5th positions of benzoic acid hydrazide and 3th and 5th positions of benzylidene fragments were observed at 6.84-7.52 ppm as multiplet. The aromatic protons belonging to 2nd and 6th positions of both benzoic acid hydrazide and benzylidene fragments gave multiplet at 7.79-8.06 ppm in all of the compounds. In the compounds **4k**-**4n**, protons of 3th and 5th positions of benzylidene fragment appeared at 8.19-8.33 ppm as a doublet. CH=N, CO-NH and N-H protons of benzimidazole appeared at 8.33-8.39 ppm, 11.71-11.76 ppm and 12.32-12.43 ppm, respectively. All the other aromatic and aliphatic protons were observed at the expected chemical shifts. All compounds gave satisfactory elemental analysis results. Mass spectra (ES-MS) of the compounds showed M+1 peaks, in agreement with their molecular formula.

Microbiology

MICs were recorded as the minimum concentration of a compound that inhibits the growth of tested microorganisms. As shown in Table I, all of the compounds were inactive against bacterial strains. However, most of the synthesized compounds indicated moderate antibacterial activity against *E. coli* when compared with the positive control chloramphenicol.

Contrary to their very poor antibacterial activity, the synthesized compounds showed significant antifungal activity against *Candida* yeasts (Table II). In the series, the compounds **4a**, **4k**, **4l**, **4m**, and **4n** seemed to be more potent to inhibit each of the *C.*



R: -H, -N(CH_3)$_2$, -N(C_2H_5)$_2$, -OH, -CH_3, -OCH_3, -OC_2H_5, -Cl, -Br, -F, -NO_2, -CF_3, -CN, -COOH.

Reagents and conditions: **a:** EtOH-benzene (5:1), catalytic amount CH_3COOH, reflux 6 h; **b:** $NaBH_4$, *i*-PrOH, reflux 2 h; **c:** 80% $NH_2NH_2.H_2O$, EtOH, reflux 12 h; **d:** corresponding 4-substitutedbenzaldehyde, catalytic amount CH_3COOH, n-BuOH, reflux 3 h.

Scheme 1. Synthetic pathway for the 4-(1H-benzimidazol-2-yl-aminomethyl)-N'-(4-substituted benzylidene) benzohydrazides

Table I. MIC values (μg/mL) of the compounds **4a-4n** against bacterial strains

Compounds	Bacterial strains								
	A	B	C	D	E	F	G	H	I
4a	25	400	400	400	200	400	100	200	400
4b	25	400	400	400	400	400	50	200	400
4c	25	400	400	400	400	400	100	400	400
4d	25	400	400	400	200	400	100	200	400
4e	25	400	400	400	200	400	100	200	400
4f	25	400	400	400	200	400	100	100	400
4g	25	400	100	400	100	400	100	200	400
4h	25	400	200	400	400	400	50	100	400
4i	25	400	100	400	200	400	100	400	400
4j	50	400	200	400	400	400	100	400	400
4k	25	400	400	400	100	400	50	400	400
4l	25	400	200	400	400	400	100	200	400
4m	25	400	200	400	400	400	100	100	400
4n	25	400	100	400	200	400	100	400	400
Chloramphenicol	12.5	12.5	50	12.5	12.5	50	12.5	12.5	12.5

A: *E. coli* 35218, **B:** *E. coli* 25922, **C:** *Proteus vulgaris,* **D:** *Salmonella thyphimurium,* **E:** *Klebsiella pneumoniae,* **F:** *Listeria monocytogenes,* **G:** *Staphylococcus aureus*, **H:** *Enterococcus faecalis,* **I:** *Bacillus subtilis*

albicans, *C. globrata* and *C. tropicalis*. Nitro substituted **4k** and cyano substituted 4 m showed two-fold better potency (MIC = 25 μg/mL) than reference drug ketoconazole (MIC = 50 μg/mL) against all of these fungal strains. Besides, antifungal activity of the **4a**, **4l** and **4n** was greater than that of the reference against *C. tropicalis*. The compounds **4l** and **4n** also showed two-fold better potency than reference against *C. albicans* and equal antifungal activity to reference against *C. globrata*. Antifungal activity of **4a** was at same degree with the reference against *C. albicans* and *C. globrata*. The other compounds (**4b**-**4j**) displayed moderate to equal antifungal activity to ketoconazole.

Table II. MIC values (μg/mL) of the compounds **4a-4n** against fungal strains

Compounds	Fungal strains		
	A	B	C
4a	50*	50*	25**
4b	50*	100	50*
4c	100	50*	50*
4d	50*	100	50*
4e	100	50*	50*
4f	50*	100	50*
4g	50*	100	50*
4h	100	50*	50*
4i	100	100	50*
4j	50*	50*	100
4k	25**	25**	25**
4l	25**	50*	25**
4m	25**	25**	25**
4n	25**	50*	25**
Ketoconazole	50	50	50

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

Achieving an important pharmacological activity is not adequate alone to develop new agents. New drug candidates also should not produce toxic effect. Thus, toxicity of the compounds **4a**, **4k**-**4n**, which have significant antifungal activity, was needed to be investigated. For this purpose, *Brine-Shrimp* lethality assay, which is considered as a useful tool for preliminary assessment of toxicity and gives reliable results in correlation with rodent and human acute oral toxicity data, was used for determination of toxicity levels of the compounds (Calleja and Persoone, 1992; Choudhary and Thomsen, 2001; Carballo et al., 2002). The LD_{50} values and 95% confidence intervals of the compounds were calculated with the LC_{50} computer program (Trimmed Spearman-Karber Method, Version 1.5) (Brayn et al., 1993). As seen in Table III, tested compounds exhibited toxicity to different extents (LD_{50} = 126.33-368.72 μg/mL). However, LD_{50} of the compounds was 3-14 folds higher than their MIC. This is a significant finding which demonstrates that the compounds display antifungal activity at non-toxic concentration. Furthermore, observation of no correlation between antifungal activity and toxic effect implies that these compounds may have selectively interacted with cell of fungi instead of host cells.

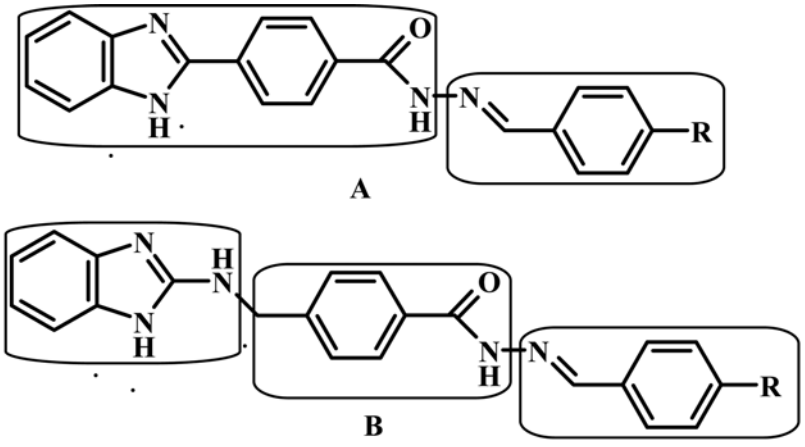
Table III. *Brine-shrimp* toxicity results of the compounds **4a, k-n**

Comp.	LD_{50} (μg/mL)	Upper 95% limit (μg/mL)	Lower 95% limit (μg/mL)
4a	368.72	688.62	222.68
4k	126.33	171.07	109.75
4l	144.26	183.50	123.46
4m	211.07	302.17	162.02
4n	193.70	351.87	134.86

Evaluation of structure-activity relationships (SARs)

In connection with our recent study (Ozkay et al., 2010), the synthesized compounds were designed to bear substituents that supply different electronic environment to the molecules. Electron donating groups to aromatic ring and electron withdrawing groups from aromatic ring were chosen for this purpose. In the recent study, we used some electronic parameters to clarify a SAR between synthesized compounds and their antibacterial activity. On the other hand, in the present study SAR was performed easier because it was very clear that potential of antifungal activity of the compounds is related with the presence of electron donating or withdrawing substituents on benzene ring. Electron withdrawing and donating groups were classified according to resonance effect constant (*R*), which is used for the substituents belonging to an aromatic ring (Hansch et al., 1973). Non-substituted compound **4a** and electron withdrawing nitro, trifluoromethyl, cyano and carboxy substituted compounds 4k-4n indicated the best antifungal activity, whereas the other compounds substituted with electron donating groups could not exhibit the same potency (Table II). The reason for above observation can be explained by electron density, which is an important factor to reach optimum antimicrobial activity. The compounds **4b-4j** possess higher electron density than the compounds **4a**, **4k-4n** since they bear electron donating groups, which enhance the electron density. It is known that high electron density causes more difficult diffusion through the cell membrane of bacteria or fungi and substantial activity loss may occur (Hania, 2009). Thus, it can be concluded that, significant antifungal activity of the compounds **4a**, **4k-4n** may be due to their reduced electron density, which makes the intracellular diffusion easier.

Another interesting finding, which should be discussed in this paper, is the antimicrobial selectivity difference between the compounds existing in the present and our recently reported (Ozkay et al., 2010) studies. In our recent study, antibacterial activity of the compounds was noteworthy, but they showed insignificant antifungal activity. On the other hand, in the present study, although the synthesized compounds exhibited low antibacterial activity, their antifungal activity was very important. The reason for such difference can be explained by following two factors: The first one is the distance between the cell structures of bacteria and yeast. For instance, while the cell wall of fungi contain chitin, the cell wall of bacteria contain murein (Eweis



Rectangle dotted tools show the conjugated areas of the compounds. (**A**) Benzimidazole-hydrazones reported in our recent study (Ozkay et al., 2010). (**B**) Benzimidazole-hydrazones (**4a-4n**) synthesized in this study. Presence of methylamino group hinders the delocalisation of π-electrons between benzimidazole ring and hydrazide moiety and causes a decrease on conjugation level.

Scheme 2. Conjugation levels of the benzimidazole-hydrazones

et al., 2006). Thus, lipophilic characters of cell membranes of the bacteria and fungi differ each other. Due to their cell nature, bacteria possess more lipophilic cell membrane than fungi (Patil et al., 2010). As a result of such distance, bacteria and fungi can show selectivity to substances during intracellular transport. The second factor is the structural distance between the benzimidazole-hydrazone series existing in the present and our recently performed studies. As seen in Scheme 2, main difference between benzimidazole-hydrazone series is the methylamine group, which exists on the chemical structure of the compounds synthesized in this study. This group possesses electron donating ability, so it hinders the delocalisation of π-electrons between benzimidazole ring and hydrazide moiety and causes a decrease on conjugation level (Scheme 2). It is known that electron delocalisation and conjugation correlate lipophilicity, which is a key property that influences the ability of a drug to reach the target by transmembrane diffusion and to have a major effect on the biological activity (Testa et al., 2000; Patil et al., 2010). As a result of their enhancing π-electron delocalisation and conjugation level, our previously synthesized benzimidazole-hydrazone derivatives would possess more lipophilicity. Depending on described two factors, it may be suggested that previously synthesized compounds possess selective antibacterial activity because of their higher lipophilicity, which may cause an easier interaction with more lipophilic cell membrane of bacteria. Otherwise, benzimidazole-hydrazone derivatives reported in this study have selective antifungal activity due to their lower lipophilic character that makes intracellular transport easier through the cell membrane of fungi.

The resistance of fungi and bacteria to current antibiotic therapies is rapidly becoming a major public health threat throughout the world in 21th century. Hence, our research group focused on the development of novel antimicrobial agents. A careful literature survey revealed that it would be worthwhile to synthesize some combination products of benzimidazole and hydrazone moieties due to their chemotherapeutic value. Depending on this purpose, we designed some compact systems, which bear these vital moieties. Our first investigation on benzimidazole-hydrazone derivatives (Ozkay et al., 2010) indicated that some of the synthesized compounds possess significant antibacterial activity. This result encouraged us and we performed the present study so as to screen antimicrobial activity of a new series of benzimidazole-hydrazones.

In the present study, some of the synthesized benzimidazole-hydrazone derivatives exhibited notable antifungal activity. Furthermore, in toxicity assay, determination of 3-14 folds higher LD_{50} than MIC was a significant result, which demonstrates that the compounds display antifungal activity at non-toxic concentration. Determination of antimicrobial selectivity difference between the compounds existing in the present and our recently reported studies was an important finding that indicates the effect of structural features on antimicrobial activity. Consequently, results of the present study have highlighted antimicrobial potentials of both benzimidazole and hydrazone moieties once more and has directed us to develop new synthesis strategies. In further studies, we believe that depending on the findings of this study it will be probable to synthesize new and more active benzimidazole-hydrazones, which display selective antifungal or antibacterial activity.

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