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Design, synthesis, characterization and pharmacological screening of some novel 2 substituted and 1(h)-substituted Benzimidazole derivatives

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***Abstract*---**The most of drugs containing Benzimidazole ring is a prominent structural motif found in numerous therapeutically active compounds. Benzimidazole and its synthetic analogues have been found to exhibit industrial, agricultural and biological application such asantitubercular, anti-inflammatory, analgesic, anticancer, anticoagulant, as well as good antifungal and anti-microbial activity.Recent advances in technology considers microwave irradiation energy as the most efficient means of heating reactions for chemical transformations that can be accomplished in a minutes. Microwave irradiation assists organic synthesis (MAOS) not only help in implementing green chemistry but also led to progress in organic synthesis. We report pharmacological screening of some novel 2 substituted and 1(h)-substituted Benzimidazole derivatives.

***Keywords*---**benzimidazole, anticancer, QSAR, characterization, pharmacologoical activity.

Introduction

The benzofused nitrogen-containing heterocyclic compounds have a great importance in drug discovery, among them benzimidazole scaffold is of particular

interest and has been categorized as a privileged scaffold.[1] The benzimidazole moiety is structurally correlated to the purine bases and is profusely found in a variety of natural products including vitamin B12.2 Several benzimidazole derivatives exhibit diversified pharmaceutical properties such as antimicrobial, [3] anticancer, [4] antiviral, [5]antihelmintic,[6] antioxidant,[7] antiulcer,[8] antihypertensive [9] and antitubercular.[10] For example, benzimidazoloquinolinone conjugate "Dovitinib (I)", a potent EGFR-3 inhibitor, is currently in phase-III clinical trials for the treatment of metastatic renal cell cancer, [11] the thiazolobenzimidazole derivative "Tiabendazole (II)" acts as an anthelmintic drug, [12] and Pimobendan (III) has been used as a vasodilator for the management of heart failure in dogs. Additionally, the bisbenzimidazole containing Hoechst stain (IV) is widely used to stain DNA in fluorescence microscopy, immunohistochemistry, and flow cytometry.

The most prominent benzimidazole compound in nature is N-riosyldimethylbenzimidazole, which serves as a axial ligand for cobalt in vitamin B12. The benzimidazole and its derivatives play a very important role as a therapeutic agent e.g.antiulcer and anthelmintic drugs. Antiulcer agents and medications for acid peptic disease are commonly used drugs that rarely cause liver injury. Most agents act by inhibition of gastric acid production, neutralization of acid or protection of the gastrointestinal mucosa from acid injury. These agents are used for both prevention and therapy of duodenal and gastric ulcer disease as well as to alleviate acid reflux, esophagitis and minor upper intestinal discomforts. The most commonly used antiulcer agents are antacids such as aluminium or magnesium hydroxide (Maalox, Mylanta and many others) and calcium carbonate (Tums, Rolaids and others).

Antacids are minimally absorbed and have no known adverse effects on the liver. Antacid use may cause a minor rise in urinary pH and rarely the calcium salts cause hypercalcemia. Other antiulcer drugs include mucosal protective agents such as sucralfate and prostaglandin analogues (misoprostol). Sucralfate (Carafate and others) is a sulfated polysaccharide that becomes a viscous polymer adhering to ulcers in mucosal surfaces and aiding in healing. Carafate is not absorbed and has not been linked to liver injury. Misoprostol is a synthetic prostaglandin E1 analogue that inhibits acid secretion and aids in ulcer healing. Misoprostol is absorbed systemically, but has not been linked to liver injury, probably because its other side effects and need for four times daily dosing limit the duration and degree of exposure. The major, most potent and effective antiulcer medications are the selective histamine type 2 receptor blockers (H2 blockers) and the proton pump inhibitors (PPIs). Both classes of antiulcer medications block the pathways of acid production or secretion, decreasing gastric acidity, improving symptoms and aiding in healing of acid-peptic diseases. These are some of the most commonly used drugs in medicine and are generally well tolerated and rarely result in serious adverse events. Nevertheless, both of these classes of agents have been linked to rare instances of acute liver injury.

Materials and Methods

Chemistry

Starting materials were purchased from commercial sources and were used without further purification. Solvents were dried according to standard procedure. The reaction progress was monitored by thin layer chromatography (TLC) on aluminium sheet obtained from Merck. Silica gel 60-120 mess was used for column chromatography. Melting points were recorded on Thermomik Campbell Melting Point apparatus having an oil bath system and were uncorrected. IR spectrum was recorded on FTIR (Perkin Elmer Spectrum RX1) by preparing KBrpellets.All1H and 13C NMR were recorded on 400 MHz Varian Mercury NMR instrument. All NMR spectra were recorded in CDCl3 or DMSO-d6 solutions using TMS as an internal standard. Chemical shifts are reported in ppm (δ). Mass spectra were recorded on Agilent 7820A GC systems (SIS-Direct insertion probe) and LC-MS TOF 6520A.

Synthesis of benzimidazole-2-carboxylate derivatives (3a-e)

Different substituted/ unsubsituted1,2-diamino-benzene (10 mmol) and diethyl oxalate (12 mmol) were stirred in 100 mL of EtOH. The reaction mixture was stirred for 4h at reflux and the completion of the reaction was monitored on Thin Layer Chromatography (TLC). Finally, the reaction mixture was cooled, diluted with 200 mL of ice cold water and solid was filtered. The solid organic precipitate (55-85% yield) was dried and this crude material was taken to the next step.

Synthesis of benzimidazole-2-carbohydrazide derivatives (4a-d)

The starting material 3a (10 mmol) was taken in 50 mL ethanol solvent and allowed to stir for 10 minutes. A 6 mL mixture of H2NNH2.H2O and HCl (1:1) was prepared at 5 to 10 oC. This mixture was transferred to the RBF containing 3a solution at room temperature. The reaction mixture was refluxed for 4h. Progress of the reaction was monitored on TLC and after completion, the reaction mixture was poured on the ice water to get solid compound. After filtration and drying of the crude material (60-70% yield), it was taken to the next step.

Synthesis of 4-phenoxybenzaldehyde derivatives (7a-f)

A mixture of phenolic compound 5a (19.35 mmol) and dried K2CO3 (58 mmol) was added to 30 mL DMSO solvent. This mixture was stirred for one hour at room temperature. 4-Fluorobenzaldehyde 6 (16.13 mmol) was added to the above stirring solution and the reaction mixture was heated at 90 oC for 16h. After completion of the reaction, the mixture was poured on crushed ice and extracted with ethyl acetate (50 mL × 3 times). The organic layer was dried over sodium sulphate and then evaporated to get crude product. Pure product, pale yellow solid (65-75% yield) was obtained by silica gel column chromatography using hexane as solvent.

General procedure for the synthesis of N'-(4-phenoxybenzylidene)-1H-benzimidazole-2-carbohydrazide derivatives (8a-j)

Benzimidazole-2-carbohydrazide derivative (0.1g, 0.57 mmol) were added to 10 mL 2 necked round bottom flask containing 4-phenoxybenzaldehyde derivatives (0.51 mmol) and 0.5 mL acetic acid. Finally, the reaction mixture was stirred for 1-3 h at 80ºC. Progress of the reaction was monitored on TLC. After completion of reaction, precipitate was formed which on cooling and after filtration gave the desired product in 80-90% yield. (E)-N'-(4-(2-chlorophenoxy) benzylidene)-1H-benzo[d]imidazole-2-carbohydrazide (8a). To the stirred solution of 1H-benzo[d]imidazole-2-carbohydrazide4a(0.1g, 0.57 mmol) in EtOH (10 mL) andAcOH(0.1mL, 1.6mmol), 4-(2-chlorophenoxy)benzaldehyde 7a (0.2g, 0.51 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4a, the solvent of reaction mixture was evaporated under reduced pressure and then quenched withanice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.172gof 8a.Buff white solid; 238-240 ºC; yield 78%; IR spectra (KBr): 3451, 3191, 3076, 2951, 1619, 1578, 1503, 1442, 1260, 1123, 748cm-1; 1H NMR (300 MHz, CDCl3): δ (ppm) 8.73 (s, 1H, –N=CH–Ar), 7.92–7.09 (m, 12H, Ar–H); LC-MS: 392 [M+1] +; Elemental Analysis for C21H15ClN4O2 (390.82): C, 64.54; H, 3.87; N, 14.34 found: C, 64.04; H, 3.62; N, 13.87(E)-N'-(4-(4-chlorophenoxy)benzylidene)-1H-benzo[d]imidazole-2 arbohydrazide(8b).

To the stirred solution of 1H-benzo[d]imidazole-2-carbohydrazide4a(0.1g, 0.57 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(4-chlorophenoxy) benzaldehyde 7b (0.2g, 0.51 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4a, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.168g of 8b. Buff white solid; 224-226 ºC; yield 76%; IR spectra (KBr): 3422, 3182, 3066, 2951, 1615, 1575, 1503, 1482, 1236, 748cm-1; 1H NMR (300 MHz, CDCl3): δ (ppm) 8.71 (s, 1H, –N=CH–Ar), 7.95–7.06 (m, 12H, Ar–H); LC-MS: 391.89 [M+1] +; Elemental Analysis for C21H15ClN4O2 (390.82): C, 64.54; H, 3.87; N, 14.34 found: C, 64.14; H, 3.57; N, 14.01.(E)-N'-(4-(4-chloro-3-methylphenoxy)benzylidene)-1H-benzo[d]imidazole-2-carbohydrazide (8c).

To the stirred solution of 1H-benzo[d]imidazole-2-carbohydrazide4a(0.1g, 0.57 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(4-chloro-3-methylphenoxy)benzaldehyde7c (0.13g, 0.51 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4a, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.149 g of 8c.Buff white solid; 232-234 ºC; yield 68 %; IR spectra (KBr): 3413, 3172, 3076, 2883, 1629, 1595, 1490, 1445, 1267, 1128, 623cm-1; 1H NMR (300MHz, CDCl3): δ (ppm) 8.54 (s,1H,–N=CH–Ar), 7.88–6.92 (m, 11H, Ar–H), 2.41(s, 3H, – CH3); LC-MS: 405.84 [M+1] +; Elemental Analysis for C22H17ClN4O2 (404.85): C, 65.27; H, 4.23; N, 13.84 found: C, 65.04; H, 3.95; N,

13.42. (E)-N'-(4-(2, 4-dichlorophenoxy) benzylidene)-1H-benzo[d]imidazole-2-carbohydrazide (8d).

To the stirred solution of 1H-benzo[d]imidazole-2-carbohydrazide4a(0.1g, 0.57 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(2, 4-dichlorophenoxy)benzaldehyde7d (0.14g, 0.51 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4a, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.173 g of 8d.Buff white solid; 235-237 °C; yield 72%; IR spectra (KBr): 3422, 3182, 3066, 2951, 1625, 1575, 1503, 1482, 1236, 748cm-1; 1H NMR (300 MHz, CDCl3): δ (ppm) 8.68 (s,1H, –N=CH–Ar), 7.95–7.06 (m, 11H, Ar–H); LC-MS: 426.2 [M+1] +; Elemental Analysis for C21H14Cl2N4O2 (425.27): C, 59.31; H, 3.32; N, 13.17 found: C, 58.91; H, 3.02; N, 12.94.(E)-N'-(4-(3,5-dichlorophenoxy)benzylidene)-1H-benzo[d]imidazole-2-carbohydrazide (8e)

To the stirred solution of 1H-benzo[d]imidazole-2-carbohydrazide4a(0.1g, 0.57 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(3,5-dichlorophenoxy)benzaldehyde7e (0.14g, 0.51 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4a, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.166 g of 8e.Buff white solid; 234-236 °C; yield 69 %; IR spectra (KBr): 3441, 3134, 3047, 2941, 2854, 1611, 1560, 1502, 1448, 1251, 1127 cm-1; 1H NMR (300 MHz, CDCl3): δ 8.56 (s, 1H, –N=CH–Ar), (ppm) 8.02–6.89 (m, 11H, Ar–H); LC-MS: 425.8 [M+1] +; Elemental Analysis for C21H14Cl2N4O2 (425.27): C, 59.31; H, 3.32; N, 13.17 found: C, 58.91; H, 3.02; N, 13.10.(E)-N'-(4-(4-methoxyphenoxy)benzylidene)-1H-benzo[d]imidazole-2-carbohydrazide(8f)

To the stirred solution of 1H-benzo[d]imidazole-2-carbohydrazide4a(0.1g, 0.57 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(4-methoxyphenoxy)benzaldehyde7f (0.12g, 0.51 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4a, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.175 g of 8f.Buff white solid; 222-224 °C; yield 80 %; IR spectra (KBr): 3432, 3193, 3085, 2989, 1627, 1587, 1465, 1364, 1250, 1127cm-1; 1H NMR (300 MHz, CDCl3): δ (ppm) 8.64 (s, 1H, –N=CH–Ar), 7.98–7.08 (m,12H, Ar–H), 3.84 (s,3H, –OCH3); LC-MS: 386.4 [M+1] +; Elemental Analysis for C22H18N4O3 (386.40): C, 68.38; H, 4.70; N, 14.50 found: C, 68.13; H, 4.35; N, 14.10. (E)-N'-(4-(naphthalen-1-yloxy)benzylidene)-1H-benzo[d]imidazole-2-carbohydrazide(8g).

To the stirred solution of 1H-benzo[d]imidazole-2-carbohydrazide4a(0.1g, 0.57 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(naphthalen-1-yloxy)benzaldehyde7g (0.13g, 0.51 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 %

EtOAc: Hexane). After consumption of 4a, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.152 g of 8g.Buff white solid; 221-223 ºC; yield 66%; IR spectra (KBr): 3442, 3182, 3067, 2955, 1620, 1565, 1490, 1434, 1280, 1128, 748cm-1; 1H NMR (300 MHz, CDCl3): δ (ppm) 8.46 (s,1H, –N=CH–Ar), 8.25–7.26 (m, 15H, Ar–H); LC-MS: 407.2 [M+1] +; Elemental Analysis for C25H18N4O2 (406.66): C, 73.88; H, 4.46; N, 13.78 found: C, 73.35; H, 4.22; N, 13.47. (E)-N'-(4-(2-chlorophenoxy)benzylidene)-6-methyl-1H-benzo[d]imidazole-2-carbohydrazide (8h) To the stirred solution of 6-methyl-1H-benzo[d]imidazole-2-carbohydrazide4b(0.1g, 0.53 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(2-chlorophenoxy)benzaldehyde7a (0.11g, 0.48 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4b, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.166 g of 8h.Buff white solid; 227-229 ºC; yield 78%; IR spectra (KBr): 3451, 3191, 3076, 2951, 1618, 1578, 1503, 1442, 1260, 1123, 748cm-1; 1H NMR (300 MHz, CDCl3): δ (ppm) 8.73 (s, 1H, –N=CH–Ar), 7.92–7.09 (m, 11H, Ar–H), 2.43 (s, 3H, – CH3); LC-MS: 406 [M+1] +; Elemental Analysis for C22H17ClN4O2 (404.85): C, 65.27; H, 4.23; N, 13.84 found: C, 65.07; H, 4.12; N, 13.77 (E)-N'-(4-(4-chlorophenoxy)benzylidene)-6-methyl-1H-benzo[d]imidazole-2-carbohydrazide(8i).

To the stirred solution of 6-methyl-1H-benzo[d]imidazole-2-carbohydrazide4b(0.1g, 0.53 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(4-chlorophenoxy)benzaldehyde7b (0.11g, 0.48 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4b, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.149 g of 8i.Buff white solid; 234-236 ºC; yield 70%; IR spectra (KBr): 3422, 3182, 3066, 2951, 1621, 1575, 1503, 1482, 1236, 748cm-1; 1H NMR (300 MHz, CDCl3): δ (ppm) 8.72 (s,1H, –N=CH–Ar), 7.95–7.06 (m, 11H, Ar–H), 2.43 (s, 3H, – CH3); LC-MS: 406 [M+1] +; Elemental Analysis for C22H17ClN4O2 (404.85): C, 65.27; H, 4.23; N, 13.84 found: C, 65.12; H, 4.03; N, 13.63 (E)-N'-(4-(2,4-dichlorophenoxy)benzylidene)-6-methyl-1H-benzo[d]imidazole-2-carbohydrazide(8j)

To the stirred solution of 6-methyl-1H-benzo[d]imidazole-2-carbohydrazide4b(0.1g, 0.53 mmol) in EtOH (10 mL) and AcOH (0.1mL, 1.6mmol), 4-(2,4-dichlorophenoxy)benzaldehyde7c (0.13g, 0.48 mmol) was added. The reaction mixture was stirred for 3 h at 80 oC. The reaction completion was monitored by TLC (50 % EtOAc: Hexane). After consumption of 4b, the solvent of reaction mixture was evaporated under reduced pressure and then quenched with an ice cold water. Precipitate formed is filtered off, washed, crystallized with EtOH and dried to afford 0.161 g of 8j.Buff white solid; 229-231 ºC; yield 70 %; IR spectra (KBr): 3441, 3210, 3085, 2970, 1625, 1585, 1506, 1460, 1261, 1133, 711cm-1; 1H NMR (300MHz, CDCl3): δ (ppm) 8.70 (s, 1H, –N=CH–Ar), 7.94–7.04 (m, 10H, Ar–H), 2.43 (s, 3H, – CH3); LC-MS: 439 [M+1] +; Elemental Analysis for

C22H16Cl2N4O2 (439.29): C, 60.15; H, 3.67; N, 12.75 found: C, 59.85; H, 3.51; N, 12.67.

SAR of N'-(4-aryloxybenzylidene)-1H-benzimidazole-2-carbohydrazide derivatives

From the biological screening of no substituted, alkyl, aroyl, nitro or halogensubstituted derivatives of benzimidazole, it was found that benzimidazole derivative with alkyl (methyl) substitution shows better activity than the aroyl (benzoyl), nitro or halogen (chloro) substitution. Methyl substituted benzimidazole derivatives 8h-j shown the activity range from 1.5 to 12.5 µg/mL, Introduction of electron donating (methyl) had a detrimental effect on the inhibitory activity compared to 8a-e.Replacement of sulphur atom in the benzothiazole ring by –NH (isostere of sulphur atom) and insertion of carbonyl group in the final designed molecule may be responsible for enhanced activity.

SAR related to the substitution on diphenyl ether moiety

Compounds having electron withdrawing (chloro) substituent on diphenyl ether moiety exhibited better activity (1.5 µg/mL), whereas other compounds showed good activity (3.125-12.5 µg/mL) except 8e. It has been observed that parachloro substitution on the diphenyl ring has a strong influence on the spectrum and extent of activity than orthosubstitution. Further, bicyclic aromatic ring (naphthalene) system was incorporated as in 8g in the diphenyl ring. The compounds containing bicyclic aromatic ring in the diphenyl ether moiety showed lower activity as compared to the monocyclic aromatic ring.

Table 1
QSAR study of Benzimidazole compounds

Title	#stars[a]	QPlogPo/w[b]	QPPCaco[c]	% Human oral absorption[d]	No of variation[e]
8a	2	4.693	781.498	100	0
8b	3	4.827	781.556	100	0
8c	2	5.075	781.541	95.482	1
8d	3	5.185	781.505	96.126	1
8e	3	5.317	781.582	96.896	1
8f	1	4.439	781.548	100	0
8g	3	5.164	781.454	96.002	1
8h	2	5	779.383	95.021	1
8i	1	5.136	781.867	95.841	1
8j	1	5.495	781.819	100	1

- a#Stars: #stars are MW, dipole, IP, EA, SASA, FOSA, FISA, PISA, WPSA, PSA, volume, donorHB, accptHB, QPlogPoct, QPlogPw, QPlogPo/w, logS, QPLogKhsa, QPlogBB. The range predicted for this parameter using QikProp is 0–5; where 0–1 indicates no violation or best candidate.
- bQPlogPo/w: This gives the predicted octanol/water partition coefficient. The range predicted for this parameter using QikProp is - 2.0–6.5.

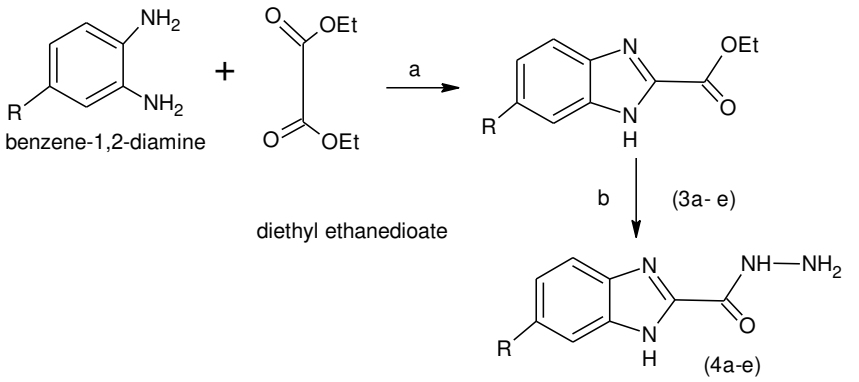
- cQPPCaco: QikProp predictions are for nonactive transport, where <25 is considered poor and >500 is considered excellent.
- d% Human-Oral Absorption: This gives the predicted human oral absorption on 0–100% scale where >80% is considered high and <25% is considered
- eNo of variation from rule of five: This property denotes the number of violations of Lipinski's rule of five

Table 4. QikProp analysis data of synthesized N'-(4-aryloxybenzylidene)-1H-benzimidazole-2-carbohydrazide derivatives

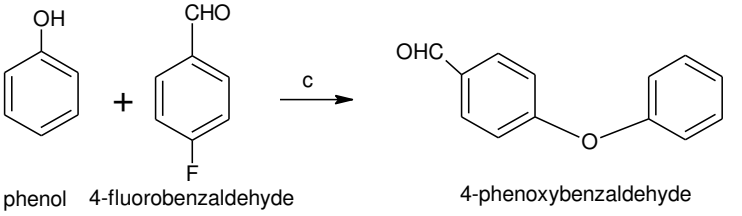
QikProp analysis

From the QikPropanalysis of designed molecules, it was observed that the designed molecules exhibited good drug likeliness (Table 4). Most of the molecules exhibited physicochemical properties which fall in the range of known drugs as evidenced from # stars for compounds being 1 or 2 (QikProp. 2013). Molecules also lacked known toxicophores or reactive functional groups in all cases. The partition coefficient exhibited by QPlogPo/w were within the range 3.5–6.1. It was observed that most of the designed molecules exhibited QPPCaco within acceptable limits (8a-j and exhibited excellent value of QPPCaco). Human oral absorption of most of the compound is >80, which was considered to be good. Most of the designed molecules also followed the Lipinski's rule of five.

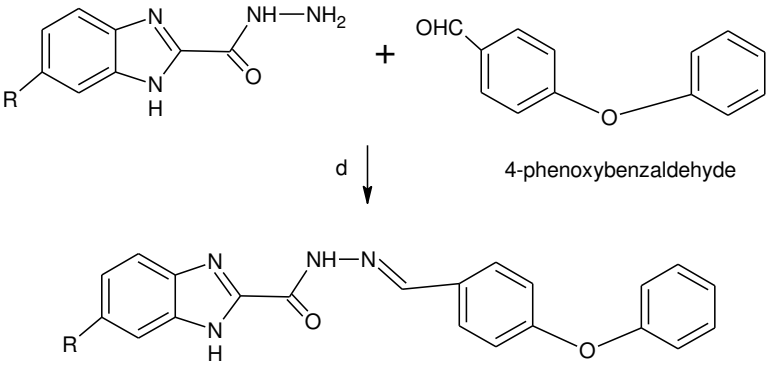
Ste.1 **Synthesis of benzimidazole-2-carboxylate derivatives**



Step 2. **Synthesis of benzimidazole-2-carbohydrazide derivatives**



Step 3. **Synthesis of 4-phenoxybenzaldehyde derivatives**



a) EtOH, Reflux 6h

b) H_2NNH_2. H_2O: HCl (1:1) ethtlene glycol reflux

c) K_2CO_3, DMSO 90^0C 16 hr.

d) EtOH, Acetic acid, reflux 2 hr

Figure 1. Scheme for synthesis of 4-phenoxybenzaldehyde

Results and Discussions

Protocol for antitubercular evaluation (REMA assay)

Resazurin Microtiter Assay (REMA), a colorimetric method for detecting drug activity against bacteria is based on the reduction of an oxidation-reduction indicator (Resazurin, Fig. 1). Activity is detected by a change in color of the

oxidation-reduction indicator, which is directly proportional to the number of viable bacteria in the medium. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. MIC of each drug is interpreted as the lowest concentration of the compound that prevents a change in color of the resazurin.

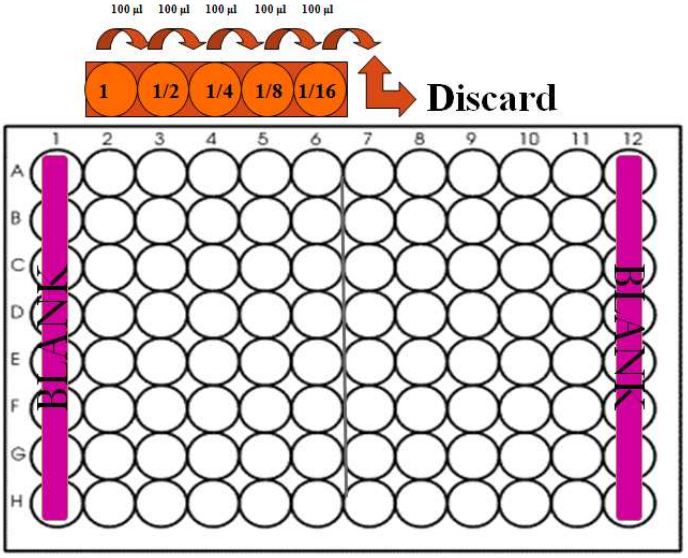


Figure 2. Diagrammatic presentation of 96 well plate preparation

All 10 novel (E)-N'-(4-aryloxybenzylidene)-*1H*-benzimidazole-2-carbohydrazide derivatives were evaluated for its *in-vitro*antitubercular activity against *M. tb* H37Rv strain using Resazurin microtitre plate assay (REMA) (Palomino et al., 2002). All compounds were evaluated for its antitubercular activity at a concentration of 1.56 to 50 µg/ml. The serial dilution technique was used to find out the minimum inhibitory concentration (MIC) of the synthesized benzimidazole derivatives. Isoniazid was used as the standard drug. Resazurin Microtiter Assay (REMA) protocol was used to determine the minimum inhibitory concentration (MIC) of the entire synthesized E)-N'-(4-aryloxybenzylidene)-*1H*-benzimidazole-2-carbohydrazidederivatives (Palomino et al., 2002). The synthesized compounds were screened against *M. tb.* H37Rv using serial dilution technique in middle brook 7H9 broth medium. Each compound (2 mg) was dissolved in 1 ml of DMSO. The serial dilution of each compounds were prepared using 96-well microtitre plate and 100 µl of *M. tb.* H37Rv cell suspension in nutrient media was added to each well. After 7 days of incubation, resazurin dye solution (0.02% w/v dissolved in distilled water) was added to each well and again incubated for 1 day. The MIC was determined by minimum concentration of compound that inhibits the growth of *M. tb.*that is indicated by colour change from non-fluorescent blue to fluorescent pink colour. The MIC values were calculated by visual inspection for each well showing more than 90 % inhibition. Isoniazid (INH) was used as the reference drug.

Antibacterial evaluation

REMA protocol was used to determine the MIC of all the synthesized (E)-N'-(4-aryloxybenzylidene)-*1H*-benzimidazole-2-carbohydrazide derivatives. The results of biological screening are summarized in Table 1. Telvekar, et al, 2012 have reported most active benzothiazole derivatives with 2,4-dichlorobiphenyl ether moiety with antitubercular activity of 1.5 µg/mL In another finding, Bairwa, et al, 2013 have reported benzothiazole derivatives 10v with pyridin-4-yl moiety with antitubercular activity of 1.35 µg/mL. Based on these findings, we had predicted that hybrid of 2,4-dichlorobiphenyl ether moiety with benzimidazole may give good antibacterial activity. However benzimidazole derivative 8j with 2,4-dichlorobiphenyl ether moiety was active at 3.125 µg/mL. In the remaining compounds 8b, 8c, 8h, 8i and 8j were having good antibacterial activity of 3.125 µg/mL against *M. tb* H37Rv strain. And also we cannot ignore the compounds 8a, 8f which has shown antitubercular activity of 6.25 µg/mL. The Benzimidazole derivatives containing Naphthyl group were least active and it may be due to presence of bulky nature of Naphthyl ring.

Table 2
Antibacterial activities of synthesized N'-(4-aryloxybenzylidene)-1H-benzimidazole-2-carbohydrazide derivatives

Comp	R	R_1	R_2	R_3	R_4	[a]MIC (µg/mL)
8a	H	Cl	H	H	H	6.25
8b	H	H	H	Cl	H	3.125
8c	H	H	Me	Cl	H	3.125
8d	H	Cl	H	Cl	H	12.5
8e	H	H	Cl	H	Cl	25
8f	H	H	H	MeO	H	6.25
8g	H	Naphthyl group				12.5
8h	Me	Cl	H	H	H	3.125
8i	Me	H	H	Cl	H	3.125
8j	Me	Cl	H	Cl	H	3.125
Standard						0.40

[a] MIC= minimum inhibitory concentration

Table 3
Antifungal activities of synthesized N'-(4-aryloxybenzylidene)-1H-benzimidazole-2-carbohydrazide derivatives

Comp	R	R_1	R_2	R_3	R_4	[a]MIC (µg/mL)
8a	H	Cl	H	H	H	12.5
8b	H	H	H	Cl	H	6.25
8c	H	H	Me	Cl	H	12.5
8d	H	Cl	H	Cl	H	12.5
8e	H	H	Cl	H	Cl	25
8f	H	H	H	MeO	H	3.25

8g	H	Naphthyl group				6.25
8h	Me	Cl	H	H	H	3.125
8i	Me	H	H	Cl	H	6.25
8j	Me	Cl	H	Cl	H	3.25
Standard						

[a] MIC= minimum inhibitory concentration

Table 4
Anticancer activities of synthesized N'-(4-aryloxybenzylidene)-1H-benzimidazole-2-carbohydrazide derivatives

Comp	R	R_1	R_2	R_3	R_4	[a]MIC (µg/mL)
8a	H	Cl	H	H	H	
8b	H	H	H	Cl	H	
8c	H	H	Me	Cl	H	
8d	H	Cl	H	Cl	H	
8e	H	H	Cl	H	Cl	
8f	H	H	H	MeO	H	
8g	H	Naphthyl group				
8h	Me	Cl	H	H	H	
8i	Me	H	H	Cl	H	
8j	Me	Cl	H	Cl	H	

[a] MIC= minimum inhibitory concentration

MTT Assay

Cells were incubated at a concentration of 1 × 10^4cells/ml in culture medium for 24 h at37°C and 5% CO2.Cells were seeded at a concentration (70µl) 10^4cells/well in 100 µl culture medium and 100µl herbal extracts into micro plates respectively (tissue culture grade, and 96 wells). Control wells were incubated with DMSO (0.2% in PBS) and cell line. All samples were incubated in triplicate. Controls were maintained to determine the control cell survival and the percentage of live cells after culture. Cell cultures were incubated for 24 h at 37°C and 5% CO2 in CO2 incubator. After incubation, the medium was completely removed and Added 20 µl of MTT reagent (5mg/min PBS). After addition of MTT, cells incubated for 4 hrs at 37oC in CO2incubator. Observed the wells for formazan crystal formation under microscope. The yellowish MTT was reduced to dark colouredformazan by viable cells only. After removing the medium completely. Added 200µlof DMSO (kept for 10 min) and incubate at 37^0C (wrapped with aluminium foil). Samples were analyzed by measuring the absorbance of each sample by a microplate reader at a wavelength of 550 nm and calculate percent inhibition by following formula % inhibition = control absorbance – test absorbance / control absorbance. (Table 4)

Table 5
Effects of compound against Hep G2 Cell line (liver cancer cell line) by MTT assay

Sr. no.	Sample	ABS T1	Percentage of cell viability	Percentage of cell inhibition	IC 50
1.	Control	0.603	--	--	--
2.	Sample 1 200 µg/ml	0.553	91.71	8.29	502.42
3.	400 µg/ml	0.518	85.91	14.09	
4.	600 µg/ml	0.502	83.26	16.74	
5.	800 µg/ml	0.450	74.63	25.37	
6.	1000 µg/ml	0.161	26.70	73.30	

Conclusion

The present study revealed that (E)-N'-(4-aryloxybenzylidene)-*1H*-benzimidazole-2-carbohydrazidederivatives possess good to moderate activity as antibacterial and antifungals. Out of 10 synthesized benzimidazole derivatives, compounds (8b-c, 8h-j) possessed excellent activity (1.5-3.125 µg/mL). The promising activity of the (E)-N'-(4-aryloxybenzylidene)-*1H*-benzimidazole-2-carbohydrazidederivatives established them as crucial pharmacophore which can be used as lead to design further novel derivatives of benzimidazole with better antibacterial and antifungal activity. Considering this pharmacophore, the further expansion of the benzimidazole series is underway to find a potent antitubercular agent. The encouraging results from the antibacterial and antifungal studies impelled us to go for preliminary screening of synthesized molecules against *Mycobacteriumtuberculosis.* Due to the better activity, some compound has been selected for further development and studies to acquire more reliable information about structure-activity relationship are in progress in our laboratory.

Thus, these compounds could act as a good potential lead for further development of new antitubercular drugs. Based on these literature reports, we found that benzimidazole is a unique as drug candidate scaffold in bacterial and fungal study. Moreover these observations indicate that the antibacerial activity depends not only on the carbohydrazide part but also on the functionalization of the carbonyl part of the benzimidazole derivatives. Importantly, substituents at the benzene ring of the benzimidazole also play a crucial role in the biological activities. In summary, benzimidazole derivatives are regarded as potent antibacterial and antifungal agents. Surprisingly, in spite of their relevant anti-TB activities, little is still known about the mode of action and understanding of the implicated molecular mechanisms. We hope to learn more about these versatile molecules and its derivatives in addition to the synthesis of new, useful biologically important compounds in the near future.

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