CHAPTER-2

Synthesis, Characterization and Biological evaluation of 1-alkyl/aralkyl-2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio) -1H-benzo[d]imidazole
Chapter Abstract

A series of pyridine conjugated benzimidazole derivatives was synthesized and evaluated for antibacterial, antioxidant and anti-inflammatory activities. The results showed that most of the tested compounds exhibited good to moderate antimicrobial activity against some strains of Gram negative bacteria (Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Shigella flexineri) and Gram positive bacteria- Bacillus subtilis. Further, the molecules were evaluated for antioxidant assays such as DPPH scavenging, superoxide radical scavenging and hydroxyl radical scavenging assays. Most of the compounds showed potent antioxidant activities. Also, the synthesized compounds were screened for anti-inflammatory activities such as lipoxygenase inhibition and indirect haemolytic assays, where these compounds revealed good activity.

2.1 Introduction

Benzimidazole derivatives have been evolved as an important heterocyclic system due to their presence in a wide range of bioactive compounds associated with wide range of biological activities such as antimicrobial,\(^\text{1-3}\) antioxidant,\(^\text{4-5}\) anti-inflammatory,\(^\text{6-8}\) antimalarial,\(^\text{9}\) antihelminthic,\(^\text{10}\) anticoagulant,\(^\text{11}\) antiamoebic,\(^\text{12}\) antihypertensive,\(^\text{13}\) lipid modulating,\(^\text{14-16}\) antidiabetic,\(^\text{17}\) antipsychotic,\(^\text{18}\) bronchodialatery\(^\text{19}\) and analgesic.\(^\text{20}\)
During the past decades, the increase in resistance by life-threatening, multidrug resistant bacteria has attracted considerable interest towards the development of new class of antimicrobial agents.\textsuperscript{21} In this direction, many research groups are engaged in developing new benzimidazole derivatives as antimicrobial agents. Iwahi and Satoh have reported some pyridine conjugated benzimidazole derivatives as antibacterial against \textit{Campylobacter pylori}.\textsuperscript{22} Johnston and coworkers have modified this pyridine conjugated benzimidazoles as omeprazole equivalents which are antibacterial against \textit{C. pylori}.\textsuperscript{23} Thereafter, many benzimidazole derivatives are being explored as potent antimicrobial agents.\textsuperscript{24}

Exposure to various organic compounds including a number of environmental pollutants and drugs can lead to the formation of reactive oxygen species such as superoxide radicals, hydroxyl radicals and hydrogen peroxide which may cause great damage to cell membranes and DNA, including oxidation that causes membrane lipid peroxidation, decreased membrane fluidity and genetic mutations which lead to cancer and other diseases.\textsuperscript{25-26} Thus, lack of antioxidant capacity of an organism may result in cancer development. As a result, various drugs possessing antioxidant and free radical scavenging activities have been implemented for the treatment of cancer. In particular many benzimidazole derivatives and their complexes with Cobalt and Copper have been reported as good anti oxidants.

Inflammation has proved to be associated with numerous diseases such as Alzheimer’s disease, asthma, atherosclerosis, carcinoma, multiple sclerosis, osteoarthritis, rheumatoid arthritis, gout, diabetes mellitus, bacterial or viral infections, etc., which result in chronic inflammation.\textsuperscript{27-28} As a consequence many ways for the control of inflammation have
been reported.\textsuperscript{29-31} A large number of compounds with diverse heterocyclic nuclei are reported to inhibit inflammation at various stages. In particular, many groups are actively engaged in developing benzimidazole derivatives as anti-inflammatory agents. However, no such molecule has made its way to the clinics so far. But, numbers of compounds targeting kinases are currently undergoing clinical trials which are related to inflammation.\textsuperscript{32}

As a part of our ongoing research program on synthesis and biological evaluation of new heterocycles,\textsuperscript{33-36} we aimed at the design and development of novel pyridine conjugated benzimidazole derivatives and screening them for antibacterial, anti-oxidant and anti-inflammatory activities, and report the results of these studies in this Chapter.

2.2 Synthesis of benzimidazole derivatives

The requisite key intermediate 7 was prepared according to the earlier reported route\textsuperscript{37} as shown in Scheme 1. The compound 2-cyano-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine 2 was prepared by nucleophilic substitution of nitro group in 3-methyl-4-nitropyridine-2-carbonitrile 1 by sodium trifluoro ethoxide. This was converted to 3-methyl-4-(2,2,2-trifluoroethoxy)-2-pyridinecarboxylic acid 3 by oxidative hydrolysis reaction with sodium nitrite in sulphuric acid. Esterification of compound 3-methyl-4-(2,2,2-trifluoroethoxy)-2-pyridinecarboxylic acid 3 with methanol in presence of sulfuric acid gave methyl 3-methyl-4-(2,2,2-trifluoroethoxy)-2-pyridinecarboxylate 4. Reduction of ester functionality in 4 by sodium borohydride in methanol afforded 2-hydroxymethyl-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine 5. Further, refluxing 5 with thionyl chloride in dichloromethane gave 2-chloromethyl-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine 6. The key intermediate compound 2-[(2-(3-methyl-4-(2,2,2-trifluoroethoxy) pyridyl)methyl]-
thio]-1H-benzimidazole 7 was obtained by reacting 2-chloromethyl-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine 6 with 2-mercaptobenzimidazole. N-alkylation of the key intermediate using various alkylating agents furnished final compounds 9a-l.

The structures of all the synthesized compounds were determined by spectral and microanalytical analyses. All compounds showed characteristic singlet between 2.3 and 2.4 δ which corresponds to pyridyl methyl protons, a quartet between 4.4 and 4.5 δ which corresponds to -OCH$_2$CF$_3$ protons and doublets at 8.30-8.40 and 7.10-7.20 due to pyridine protons in $^1$H NMR spectra which confirms the formation of products. The structures and yields of the synthesized compounds are given in Table 1.

Reagents and condition: i) NaOCH$_2$CF$_3$ ii) NaNO$_2$, H$_2$SO$_4$ iii) H$_2$SO$_4$, CH$_3$OH iv) NaBH$_4$, Methanol v) SOCl$_2$, DCM vi) Mercaptobenzimidazole, Methanol vii) 8a-l, Toluene, 30% NaOH, TBAB

Scheme 1
Table 1. Structures of synthesized compounds and yields

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2.3 Experimental Section

The melting points were determined on Selaco melting point apparatus and are uncorrected. Infrared spectra were recorded on Shimadzu FT-IR model 8300 spectrophotometer. $^1$H NMR spectra were recorded on an NMR spectrometer operating at 400MHz using TMS as internal standard. Mass spectra were recorded using electrospray ionization mass spectrometry. The C, H and N analysis were performed using CE-400 CHN analyzer. Reactions were monitored by TLC using precoated sheets of silica gel G/UV-254 of 0.25 mm thickness (Merck 60F254) using UV light for visualization.

All chemicals were obtained from Aldrich, Fluka and Merck Chemicals.

2.3.1 General Procedure for the synthesis of compounds 9a-l.

To a solution of pyridine conjugated benzimidazole 7 (10 mmol) and tetrabutyl ammonium bromide (1 mmol) in toluene (20 mL), 50% NaOH solution (25 mL) was added at 0°C followed by the addition of alkylating agents 8a-l (12mmol). The reaction mixture was stirred vigorously at room temperature for 6-10h and the reaction was monitored by TLC. After the completion of the reaction, aqueous phase was separated and the organic phase was washed with water (20mL) and brine (20mL), dried over
anhydrous sodium sulphate and concentrated to give crude products which were purified by column chromatography over silica gel using hexane-EtOAc (6:4) mixture as eluent.

2.3.2 Characterization data

1-Benzyl-2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio)-1H-benzo[d]imidazole (9a):

White solid; mp. 90-92°C; IR (KBr) cm⁻¹: 2943 (C-H aromatic), 1693 (C=C aromatic); ¹H-NMR (CDCl₃, 400 MHz) δ 2.34 (s, 3H, CH₃), 4.40 (q, 2H, J=8.0 Hz, CH₂CF₃), 4.89 (s, 2H, CH₂), 5.29 (s, 2H, CH₂), 7.04-7.32 (m, 9H, Ar-H), 7.73 (d, 1H, J=8.0 Hz, Ar-H), 8.34 (d, 1H, J=6.0 Hz, Ar-H); ¹³C NMR (CDCl₃, 100 MHz): 161.7, 155.4, 151.7, 147.8, 143.2, 136.2, 134.1, 133.9, 129.2, 128.5, 122.6, 122.5, 121.7, 118.6, 109.0, 105.6, 65.6, 65.4, 47.0, 37.4, 10.8. MS (ESI): m/z 444 (M+1); Anal. Calcd for C₂₃H₂₀F₃N₃OS: C, 62.29; H, 4.55; N, 9.47; Found: C, 62.35; H, 4.65; N, 9.56.

1-(4-Chlorobenzyl)-2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio)-1H-benzo[d]imidazole (9b):

White solid; mp. 128-130°C; IR (KBr) cm⁻¹: 2920 (C-H aromatic), 1652 (C=C aromatic); ¹H-NMR (CDCl₃, 400MHz) δ 2.34 (s, 3H, CH₃), 4.40 (q, 2H, J=8.0 Hz, CH₂CF₃), 4.88 (s, 2H, CH₂), 5.58 (s, 2H, CH₂), 6.66 (d, 1H, J=5.6 Hz, Ar-H), 7.04-7.32
(m, 7H, Ar-H), 7.73 (d, 1H, J=8.0 Hz, Ar-H), 8.34 (d, 1H, J=8.0 Hz, Ar-H); $^{13}$C NMR (CDCl$_3$, 100 MHz): 161.7, 155.5, 151.8, 147.9, 143.4, 136.0, 134.0, 133.8, 129.0, 128.3, 122.4, 122.3, 121.7, 118.5, 109.0, 105.6, 65.6, 65.3, 47.02, 37.4, 10.74; MS (ESI): m/z 478 (M+1); Anal. Calcd for C$_{23}$H$_{19}$ClF$_3$N$_3$OS: C, 57.80; H, 4.04; N, 8.79; Found: C, 57.83; H, 4.09; N, 8.86.

2-((3-Methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio)-1-(4-methylbenzyl)-1H-benzo[d]imidazole (9c):

White solid; mp. 96-98°C; IR (KBr) cm$^{-1}$: 2929 (C-H aromatic), 1584 (C=C aromatic); $^1$H NMR (CDCl$_3$, 400 MHz) δ 2.29 (s, 3H, CH$_3$), 2.32 (s, 3H, CH$_3$), 4.45 (q, 2H, J=8.0 Hz, CH$_2$CF$_3$), 4.89 (s, 2H, CH$_2$), 5.28 (s, 2H, CH$_2$), 6.65 (d, 1H, J=6.0 Hz, Ar-H), 7.00-7.30 (m, 7H, Ar-H), 7.72 (d, 1H, J=8.0 Hz, Ar-H), 8.34 (d, 1H, J=5.6 Hz, Ar-H); $^{13}$C NMR (CDCl$_3$, 100 MHz): 161.8, 155.4, 151.8, 147.7, 142.9, 137.7, 136.04, 132.3, 129.0, 127.3, 126.9, 123.2, 122.9, 118.1, 111.2, 109.6, 105.7, 65.3, 60.7, 47.6, 21.0, 7.6; MS (ESI): m/z 458 (M+1); Anal. Calcd for C$_{24}$H$_{22}$F$_3$N$_3$OS: C, 63.01; H, 4.85; N, 9.18; Found: C, 63.09; H, 4.89; N, 9.25.

1-(3-Fluorobenzyl)-2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio)-1H-benzo[d]imidazole (9d):
White solid; mp. 90-92°C; IR (KBr) cm\(^{-1}\): 2890 (C-H aromatic), 1600 (C=C aromatic); 
\(^1\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 2.34 (s, 3H, CH\(_3\)), 4.39 (q, 2H, J=8.0 Hz, CH\(_2\)CF\(_3\)), 4.87 (s, 2H, CH\(_2\)), 5.30 (s, 2H, CH\(_2\)), 6.64 (d, 1H, J=5.2 Hz, Ar-H), 6.82 (d, 1H, J=9.2 Hz, Ar-H), 6.93 (t, 2H, J=2.8 Hz, Ar-H), 7.14-7.27 (m, 4H, Ar-H), 7.72 (d, 1H, J=8.0 Hz, Ar-H), 8.34 (d, 1H, J=5.6 Hz, Ar-H); \(^13\)C NMR (CDCl\(_3\), 100 MHz): 164.2, 161.8, 155.6, 151.8, 148.0, 143.6, 138.1, 136.1, 130.05, 124.3, 122.4, 122.3, 118.7, 115.0, 114.8, 114.0, 133.7, 109.0, 105.6, 65.60, 47.09, 37.5, 10.7; MS (ESI): m/z 462 (M+1); Anal. Calcd for C\(_{23}\)H\(_{19}\)F\(_4\)N\(_3\)OS: C, 59.86; H, 4.15; N, 9.11; Found: C, 59.95; H, 4.20; N, 9.17.

1-(3,4-Dichlorobenzyl)-2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio) – 1H-benzo[d]imidazole (9e):

White solid; mp. 108-110°C; IR (KBr) cm\(^{-1}\): 2901 (C-H aromatic), 1579 (C=C aromatic); 
\(^1\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 2.34 (s, 3H, CH\(_3\)), 4.39 (q, 2H, J=8.0 Hz, CH\(_2\)CF\(_3\)), 4.86 (s, 2H, CH\(_2\)), 5.27 (s, 2H, CH\(_2\)), 6.69 (d, 1H, J=5.6 Hz, Ar-H), 6.97 (q, 1H, J=1.6 Hz, Ar-H), 7.14-7.36 (m, 6H, Ar-H), 7.72 (d, 1H, J=8.0 Hz, Ar-H), 8.34 (d, 1H, J=5.2 Hz, Ar-H); \(^13\)C NMR (CDCl\(_3\), 100 MHz): 161.6, 155.4, 151.7, 148.0, 143.5, 135.9, 135.8, 132.9, 130.8, 128.7, 126.18, 122.3, 121.9, 121.5, 121.4, 118.5, 108.9, 105.6, 65.5, 65.9, 46.4, 39.4, 10.65; MS (ESI): m/z 512 (M+1); Anal. Calcd for C\(_{23}\)H\(_{18}\)Cl\(_2\)F\(_3\)N\(_3\)OS: C, 53.91; H, 3.54; N, 8.20; Found: C, 53.97; H, 3.57; N, 8.26.
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1-(3-Methoxybenzyl)-2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio)-1H-benzo[d]imidazole (9f):

White solid; mp. 76-78°C; IR (KBr) cm⁻¹: 2943 (C-H aromatic), 1606 (C=C aromatic);

¹H-NMR (CDCl₃, 400 MHz) δ 2.35 (s, 3H, CH₃), 3.72 (s, 3H, OCH₃), 4.44 (q, 2H, J=8.0 Hz, CH₂CF₃), 4.92 (s, 2H, CH₂), 5.31 (s, 2H, CH₂), 6.69-6.80 (m, 4H, Ar-H), 6.94 (s, 1H, Ar-H), 7.18-7.29 (m, 3H, Ar-H), 7.25 (d, 1H, J=8.0 Hz, Ar-H), 8.35 (d, 1H, J=5.6 Hz, Ar-H); ¹³C NMR (CDCl₃, 100 MHz): 171.4, 162.1, 151.8, 147.4, 147.4, 143.7, 142.6, 136.9, 133.2, 130.0, 126.5, 124.3, 122.5, 119.5, 118.2, 113.2, 109.5, 105.8, 65.4, 63.42, 55.2, 47.8, 37.3, 12.14; MS (ESI): m/z 474 (M+1); Anal. Calcd for C₂₄H₂₂F₃N₃O₂S: C, 60.88; H, 4.68; N, 8.87; Found: C, 60.93; H, 4.75; N, 8.94.

2-((3-Methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio)-1-(3-methylbenzyl)-1H-benzo[d]imidazole (9g):

White solid; mp. 114-116°C; IR (KBr) cm⁻¹: 2915 (C-H aromatic), 1590 (C=C aromatic);

¹H-NMR (CDCl₃, 400 MHz) δ 2.27 (s, 3H, CH₃), 2.36 (s, 3H, CH₃), 4.40 (q, 2H, J=8.0 Hz, CH₂CF₃), 4.90 (s, 2H, CH₂), 5.29 (s, 2H, CH₂), 6.66 (d, 1H, J=5.6 Hz, Ar-H), 6.94 (s,
2H, Ar-H), 7.04 (t, 1H, J=7.6 Hz, Ar-H), 7.14-7.26 (m, 4H, Ar-H), 7.74 (d, 1H, J=8.0 Hz, Ar-H), 8.34 (d, 1H, J=5.6 Hz, Ar-H); $^1$H NMR (CDCl$_3$, 100 MHz): 161.7, 155.6, 151.9, 149.7, 143.1, 138.6, 136.2, 128.7, 124.3, 127.5, 124.3, 123.9, 122.3, 121.7, 121.5, 118.2, 109.3, 105.6, 66.0, 65.6, 47.7, 37.5, 21.3, 10.7; MS (ESI): m/z 458 (M+1); Anal. Calcd for C$_{24}$H$_{22}$F$_3$N$_3$OS: C, 63.01; H, 4.85; N, 9.18; Found: C, 63.09; H, 4.92; N, 9.24.

2-((3-Methyl-4-(2,2,2-trifluoroethox)pyridin-2-yl)methylthio)-1-(4-nitrobenzyl)-1H-benzo[d]imidazole (9h):

White solid; mp. 104-106°C; IR (KBr) cm$^{-1}$: 2885 (C-H aromatic), 1590 (C=C aromatic), 1955 (NO$_2$); $^1$H-NMR (CDCl$_3$, 400 MHz) $\delta$ 2.33 (s, 3H, CH$_3$), 4.40 (q, 2H, J=8.0 Hz, CH$_2$CF$_3$), 4.90 (s, 2H, CH$_2$), 5.30 (s, 2H, CH$_2$), 6.85 (d, 2H, J=8.0 Hz, Ar-H), 7.00-7.35 (m, 5H, Ar-H), 7.72 (d, 2H, J=8.0 Hz, Ar-H), 8.30 (d, 1H, J=8.0 Hz, Ar-H); $^{13}$C NMR (CDCl$_3$, 100 MHz): 161.7, 155.3, 151.9, 147.4, 142.3, 137.8, 136.3, 132.4, 129.3, 127.5, 126.8, 123.2, 122.6, 118.1, 111.2, 109.3, 105.4, 65.1, 60.3, 47.4, 7.6; MS (ESI): m/z 489 (M+1); Anal. Calcd for C$_{23}$H$_{19}$F$_3$N$_4$O$_3$S: C, 56.55; H, 3.92; N, 11.47; Found: C, 56.62; H, 3.99; N, 11.55.

1-Methyl-2-((3-methyl-4-(2,2,2-trifluoroethox)pyridin-2-yl)methylthio)-1H-benzo[d]imidazole (9i):
White solid; mp. 108-110°C ; IR (KBr) cm\(^{-1}\): 2887 (C-H aromatic), 1681 (C=C aromatic); \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta\) 2.34 (s, 3H, CH\(_3\)), 3.72 (s, 3H, NCH\(_3\)), 4.39 (q, 2H, J=8.0 Hz, CH\(_2\)CF\(_3\)), 4.80 (s, 2H, CH\(_2\)), 7.11 (d, 2H, J=7.8 Hz, Ar-H), 7.25-7.35 (m, 3H, Ar-H), 8.33 (d, 1H, J=8.0 Hz, Ar-H); \(^1\)C NMR (CDCl\(_3\), 100 MHz): 152.4, 147.2, 138.9, 134.2, 123.0, 123.5, 122.4, 115.2, 118.8, 110.0, 104.7, 82.8, 38.5, 32.9, 10.2; MS (ESI): m/z 368 (M+1); \textit{Anal}. Calcd for C\(_{17}\)H\(_{16}\)F\(_3\)N\(_3\)OS: C, 55.58; H, 4.39; N, 11.44; Found: C, 55.65; H, 4.46; N, 11.51.

\textbf{2-((3-Methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl) methylthio)-1-pentyl-1H-benzo[d]imidazole (9j):}

White solid; mp. 110-112°C ; IR (KBr) cm\(^{-1}\): 2919 (C-H aromatic), 1648 (C=C aromatic); \(^1\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 0.9 (t, 3H, J=8.0 Hz, CH\(_3\)), 1.29-1.32 (m, 4H, (CH\(_2\))\(_2\)), 1.74 (m, 2H, CH\(_2\)), 2.35 (s, 3H, CH\(_3\)), 3.70 (t, 2H, J=8.0 Hz, NCH\(_2\)), 4.40 (q, 2H, J=8.0 Hz, CH\(_2\)CF\(_3\)), 4.90 (s, 2H, CH\(_2\)), 7.10 (d, 2H, J=8.0 Hz, Ar-H), 7.24-7.36 (m, 3H, Ar-H), 8.32 (d, 1H, J=8.0 Hz, Ar-H); \(^1\)C NMR (CDCl\(_3\), 100 MHz): 162.0, 160.8, 152.4, 147.2, 138.9, 134.2, 123.6, 123.0, 115.4, 111.9, 1110.0, 104.7, 82.8, 49.0, 38.5, 29.2, 28.9, 22.4, 14.1, 10.1; MS (ESI): m/z 424 (M+1); \textit{Anal}. Calcd for C\(_{21}\)H\(_{24}\)F\(_3\)N\(_3\)OS: C, 59.56; H, 5.71; N, 9.92; Found: C, 59.60; H, 5.77; N, 9.98.

\textbf{1-Butyl-2-((3-methyl-4-(2,2,2-trifluoroethoxy) pyridin-2-yl) methylthio)-1H-benzo[d]imidazole (9k):}

White solid; mp. 110-112°C ; IR (KBr) cm\(^{-1}\): 2919 (C-H aromatic), 1648 (C=C aromatic); \(^1\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 0.9 (t, 3H, J=8.0 Hz, CH\(_3\)), 1.29-1.32 (m, 4H, (CH\(_2\))\(_2\)), 1.74 (m, 2H, CH\(_2\)), 2.35 (s, 3H, CH\(_3\)), 3.70 (t, 2H, J=8.0 Hz, NCH\(_2\)), 4.40 (q, 2H, J=8.0 Hz, CH\(_2\)CF\(_3\)), 4.90 (s, 2H, CH\(_2\)), 7.10 (d, 2H, J=8.0 Hz, Ar-H), 7.24-7.36 (m, 3H, Ar-H), 8.32 (d, 1H, J=8.0 Hz, Ar-H); \(^1\)C NMR (CDCl\(_3\), 100 MHz): 162.0, 160.8, 152.4, 147.2, 138.9, 134.2, 123.6, 123.0, 115.4, 111.9, 1110.0, 104.7, 82.8, 49.0, 38.5, 29.2, 28.9, 22.4, 14.1, 10.1; MS (ESI): m/z 424 (M+1); \textit{Anal}. Calcd for C\(_{21}\)H\(_{24}\)F\(_3\)N\(_3\)OS: C, 59.56; H, 5.71; N, 9.92; Found: C, 59.60; H, 5.77; N, 9.98.
White solid; mp. 88-90°C ; IR (KBr) cm\(^{-1}\): 2870 (C-H aromatic), 1585 (C=C aromatic); 
\(^1\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 0.91 (t, 3H, J=8.0 Hz, CH\(_3\)), 1.30 (m, 2H, CH\(_2\)), 1.72 (m, 2H, CH\(_2\)), 2.34 (s, 3H, CH\(_3\)), 4.02 (t, 2H, J=8.0 Hz), 4.41 (q, 2H, J=8.0 Hz, CH\(_2\)CF\(_3\)), 4.91 (s, 2H, CH\(_2\)), 7.10 (d, 2H, J=8.0 Hz, Ar-H), 7.24-7.36 (m, 3H, Ar-H), 8.31 (d, 1H, J = 8.0Hz, Ar-H); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz): 162.5, 160.8, 152.5, 147.3, 138.8, 134.2, 123.6, 123.0, 115.2, 111.8, 1110.0, 104.7, 82.8, 48.7, 38.4, 32.4, 20.1, 13.8, 10.1; MS (ESI): m/z 410 (M+1); Anal. Calcd for C\(_{20}\)H\(_{22}\)F\(_3\)N\(_3\)OS: C, 59.66; H, 5.66; N, 9.42; Found: C, 59.71; H, 5.69; N, 9.50.

**1-Heptyl-2-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methylthio)-1H-benzo[d]imidazole (9i):**

White solid; mp. 82-84°C ; IR (KBr) cm\(^{-1}\): 2932 (C-H aromatic), 1660 (C=C aromatic); 
\(^1\)H-NMR (CDCl\(_3\), 400 MHz) \(\delta\) 0.87 (t, 3H, J=8.0 Hz, CH\(_3\)), 1.29-1.32 (m, 8H, (CH\(_2\))\(_4\)),1.74 (m, 2H, CH\(_2\)), 2.34 (s, 3H, CH\(_3\)), 3.72 (t, 2H, J=8.0 Hz, NCH\(_2\)), 4.41 (q, 2H, J=8.0 Hz, CH\(_2\)CF\(_3\)), 4.92 (s, 2H, CH\(_2\)), 7.10 (d, 2H, J=8.0 Hz, Ar-H), 7.24-7.36 (m, 3H, Ar-H), 8.31 (d, 1H, J=8.0 Hz, Ar-H); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz):162.0, 160.5, 152.6, 147.0, 138.8, 134.1,123.8, 123.2, 115.3, 11.8, 110.1, 104.7, 82.8, 49.0, 38.5, 31.8, 29.2, 27.0, 22.7, 14.1, 10.2; MS (ESI): m/z 452 (M+1); Anal. Calcd for C\(_{23}\)H\(_{28}\)F\(_3\)N\(_3\)OS: C, 61.18; H, 6.25; N, 9.31; Found: C, 61.25; H, 6.31; N, 9.38.
2.4 Biological activity

2.4.1 Antibacterial Activity

Agar well diffusion assay (zone of inhibition test) is a simple satisfactory method to evaluate the effectiveness of antiseptic or chemical agent against selected test microorganisms. It measures the susceptibility of a particular microorganism to specific chemicals/antibiotics, antimicrobial agents, or even herbal extracts. The susceptibility of the microorganism to the specific antibiotic is indicated by the appearance of a clear zone surrounding the agar well (i.e., zone of inhibition). The presence of a clear zone of inhibition surrounding the well is indicative of inhibitory (antimicrobial) activity against the organism.

All newly synthesized compounds were evaluated for their antibacterial activity against five human pathogenic bacterial strains. These included *Escherichia coli* (MTCC 40), *Klebsiella pneumoniae* (MTCC 661), *Salmonella typhi* (MTCC 733), *Shigella flexneri* (MTCC 1457) and *Bacillus subtilis* (Clinical isolate).

Antibacterial tests were carried out by modified agar well diffusion method. The sterile petriplate is first labelled with the name of the microorganism to be inoculated. The nutrient agar is prepared, and sterilized by autoclave. Sterile nutrient agar is cooled to 40°C and poured into the sterile petriplate and allowed to solidify. Nutrient agar is then inoculated with 10^6 cfu/mL of respective microorganism using sterile spreader. Wells are made using sterile cork borer (8.5 mm) and compounds to be tested are added to each well with different concentrations ranging from 10 to 1000 μg/mL of synthesized compounds, which were made from the stock solution of 4 mg/mL in DMSO. A 100 μL volume of each dilution introduced into wells (in duplicates) in the agar plates already
seeded with bacterial suspension. These plates were incubated at 37°C for 24h and observed for the inhibition zones. Gentamicin and Chloramphenicol is used as positive controls. Zone of inhibition of all the compounds 9a-l was determined by the modified agar well diffusion method.

2.4.1.1 Results and Discussion

All synthesized compounds exhibited the antibacterial activity in broad spectrum. In the present study, 9a, 9c and 9f displayed prominent zone of inhibition with low minimum inhibitory concentrations against both Gram positive and Gram negative bacterial strains. 9d specifically showed good zone of inhibition against Gram positive organisms as compared to positive control. Compounds 9b, 9e and 9g showed good activity against E.coli, B.subtilis and moderate activity against other strains. 9h is found be inactive against all the strains. In brief, 9a, 9c, 9d and 9f are potent candidates as antibacterial agents. The zone of inhibition and MIC of all the test compounds are tabulated in Table 2.
Table 2. Antibacterial activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Escherichia coli</th>
<th>Bacillus subtilis</th>
<th>Klebsiella pneumoniae</th>
<th>Salmonella typhi</th>
<th>Shigella flexneri</th>
</tr>
</thead>
<tbody>
<tr>
<td>9a</td>
<td>23</td>
<td>25</td>
<td>14</td>
<td>29</td>
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<tr>
<td>9b</td>
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<td>9c</td>
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<td>20</td>
<td>17</td>
<td>26</td>
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<tr>
<td>9d</td>
<td>13</td>
<td>20</td>
<td>16</td>
<td>18</td>
<td>16</td>
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<tr>
<td>9e</td>
<td>19</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>9f</td>
<td>22</td>
<td>26</td>
<td>16</td>
<td>29</td>
<td>24</td>
</tr>
<tr>
<td>9g</td>
<td>16</td>
<td>19</td>
<td>-</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>9h</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9i</td>
<td>15</td>
<td>14</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9j</td>
<td>15</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>9k</td>
<td>14</td>
<td>16</td>
<td>-</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>9l</td>
<td>15</td>
<td>14</td>
<td>15</td>
<td>12</td>
<td>14</td>
</tr>
</tbody>
</table>

* Inhibition zones including cup borer (8.5 mm) diameter

Positive control zone is 35 to 40 mm

"-" = Not active

Photograph indicating the zone of inhibition of compound 9d
2.4.2 Antioxidant activity

Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system. However, oxidative stress is generated when equilibrium favours free radical generation as a result of a depletion of antioxidant levels. Oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, such as cancer, cardiovascular diseases, neurodegenerative disorders, and ageing, thus antioxidants are considered important nutraceuticals on account of many health benefits.

2.4.2.1 DPPH radical scavenging assay

The scavenging activity for DPPH radicals was determined as described by Chuanga, et al.\(^{38}\) Assays were performed in 300 µL reaction mixtures containing 200 µL of 0.1 mM DPPH–ethanol solution, 90µL of 50mM Tris–HCl buffer (pH 7.4), and 10 µL of deionised water (as control) and with various concentrations of compounds 9a-l (0.4 - 2µg/ml). Ascorbic acid was used as standard. After 30 min of incubation at room temperature, absorbance (540 nm) of the reaction mixtures was taken by a plate reader (Lab systems Mullikan MS). The percentage radical scavenging activity was calculated according to the following formula:

\[
\text{Inhibition (\%)} = \left( \frac{\text{Absorbance control-Absorbance Sample}}{\text{Absorbance Control}} \right) \times 100
\]
The results of DPPH radical scavenging assay are summarized in Figure 1 and Table 3.

**Figure 1.** DPPH Scavenging assay

### 2.4.2.2. Hydroxyl radical scavenging assay

The hydroxyl radical (·OH) scavenging activity was determined according to the reported method of Halliwell, et al.\(^{39}\) The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe\(^{3+}\)-ascorbate-EDTA-H\(_2\)O\(_2\) system (Fenton’s reaction). The reaction mixture in final volume of 2 mL containing 0.1 mL of EDTA (1 mM), 0.01 mL of FeCl\(_3\) (10 mM), 0.1 mL of H\(_2\)O\(_2\) (10 mM), 0.36 mL of deoxyribose (10 mM), 1 mL of the compounds 9a-l (concentrations from 0.4-2 µg/ml), 0.33 mL of phosphate buffer (50 mM, pH 7.4) and 0.1 mL ascorbic acid (1 mM) added in sequence. The mixture was incubated at 37\(^\circ\)C for 1 h. One mL of the incubated mixture was mixed with 1 mL of 10% trichloro acetic acid and 1 mL of TBA (1% in 0.025 M NaOH), the resulting mixture was incubated in water bath at 90\(^\circ\)C for 20 min to develop a pink chromogen which was measured at 532 nm. BHA was used as a standard. Percentage inhibition was evaluated by comparing the test and blank solutions.
Hydroxyl radical scavenging activities of compounds are presented in Figure 2 and Table 3.

**Figure 2.** Hydroxyl radical scavenging assay

### 2.4.2.3 Superoxide anion radical scavenging assay

The superoxide anion radical scavenging activity was determined as reported by Nishimiki, et al. 40. Briefly 1 mL of NBT (156 µM NBT in 100 mM phosphate buffer of pH 7.4), 1mL of NADH (468 µM in 100 mM phosphate buffer of pH 7.4) and varying concentrations of compounds 9a-l (0.4-2 µg/ml) were mixed to give a final volume of 3 mL. The reaction was started by the addition of 100 µl of PMS (60 µM in 100 mM phosphate buffer of pH 7.4). The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm. Quercetin was used as a standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

Superoxide anion radical scavenging activities of compounds are presented in Figure 3 and Table 3.
2.4.2.4 Results and Discussion

The IC$_{50}$ values of the standards and test samples were summarized in Table-3. The results of all synthesized compounds showed DPPH scavenging activity in a dose dependent manner. In particular, 9g, 9j and 9e have exhibited significant scavenging activity as compared to standard ascorbic acid. Compounds 9a, 9b, 9c, 9h, 9k and 9l showed good scavenging activity, while 9d, 9f and 9i showed moderate scavenging activity. In hydroxyl radical scavenging assay, 9g and 9k showed significant inhibition of hydroxyl radical formation compared to standard BHA. 9c and 9e showed good hydroxyl radical scavenging activity, while remaining compounds showed considerable inhibition in a dose dependent manner. In superoxide radical scavenging assay, 9e and 9j showed significant scavenging activity compared to standard Quercetin. 9k, 9g, 9c and 9b showed good scavenging activity. Remaining compounds showed moderate activity.
Table 3. IC\textsubscript{50} values of compounds 9a-1 for antioxidant activity

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50} values in µg/mL</th>
<th>DPPH scavenging assay</th>
<th>Hydroxy radical scavenging assay</th>
<th>Superoxide radical scavenging assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>9a</td>
<td>1.5</td>
<td>1.5</td>
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<td>9b</td>
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<td>1.8</td>
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<td>9c</td>
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<td>0.7</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>9d</td>
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<td>1.2</td>
<td>1.8</td>
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</tr>
<tr>
<td>9e</td>
<td>0.44</td>
<td>0.8</td>
<td>0.35</td>
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</tr>
<tr>
<td>9f</td>
<td>2.0</td>
<td>1.9</td>
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<td>9g</td>
<td>0.36</td>
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<tr>
<td>9h</td>
<td>0.61</td>
<td>1.1</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>9i</td>
<td>2</td>
<td>1.4</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>9j</td>
<td>0.39</td>
<td>0.9</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>9k</td>
<td>1</td>
<td>0.39</td>
<td>0.40</td>
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<tr>
<td>9l</td>
<td>1</td>
<td>1</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Ascorbic Acid</td>
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<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>BHA</td>
<td>-</td>
<td>0.33</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>-</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

\(^{\ast\ast\ast}\) = Not determined

2.4.3 Anti-inflammatory activity

2.4.3.1. Lipoxygenase inhibition assay

Lipoxygenase inhibition assay was carried out using linoleic acid as substrate and lipoxigenase enzyme.\textsuperscript{41} To a solution of 0.1 mL of 0.2 M borate buffer (pH 9.0), 0.1 mL of 1000 units lipoyxidase enzyme solution and varying concentrations of compounds 9a-1 (0.3 – 1.5µg/mL) dissolved in DMSO were added. The tubes were agitated and incubated at 37\(^{\circ}\)C for 5 min, after which 2.0 mL of substrate solution, and 0.6 mM linoleic acid were added, mixed well and the absorbance was measured spectrophotometrically for 4 min at 234 nm (Shimadzu 2401 PC). Indomethacin was used as reference standard drug. Percent (%) inhibition was calculated by the following equation,

\[
\text{Inhibition (\%)} = \frac{(\text{Absorbance control} - \text{Absorbance Sample})}{\text{Absorbance Control}} \times 100
\]
A dose response curve was plotted to determine the IC$_{50}$ values.

Lipoxygenase inhibition activities are shown in Figure 4 and Table 4.

![Graph showing lipoxygenase inhibition activities](image)

**Figure 4. In vitro anti-lipoygenase activity**

2.4.3.2 Inhibition of PLA$_2$ induced haemolysis in human erythrocytes

Indirect haemolytic assay was carried out according to the method developed by Boman and Kaletta.$^{42}$ The substrate for indirect hemolytic activity was prepared by suspending 1 mL of fresh human red blood cells and 1 mL of fresh Hen’s egg yolk in 8 mL of phosphate buffered saline. One mL of suspension was incubated with 4-28 μg of partially purified venom for 45 min at 37°C and the reaction was stopped by the addition of 9 mL of ice cold PBS. The suspension was centrifuged at 2000 rpm for 20 min and then the released hemoglobin was read at 540 nm. For inhibition studies 10 μg of venom sample (secretory-PLA$_2$ purchased from sigma) were incubated with various concentrations of compounds 9a-l (1-5μg/ml dissolved in DMSO) for 30 min at room temperature and 1 mL of substrate was added, again incubated for 30 min at room temperature and the
reaction was stopped by adding 9 mL of ice cold PBS to all test tubes and centrifuged at 2000 rpm for 10 min. Finally absorbance was measured at 540 nm.

The results of PLA2 inhibition assay are summarized in Figure 5 and Table 4.

![Graph showing % of hemolysis inhibition vs concentration of test compounds](image)

**Figure 5.** Inhibition of PLA2 induced hemolysis in human erythrocytes

### 2.4.3.3 Results and Discussion

In lipoxygenase inhibition activity, compounds 9a-l showed considerable inhibition of lipoxygenase enzyme compared to the standard Indomethacin. In particular 9e and 9h have significantly inhibited the lipoxygenase activity. While 9b, 9j, 9k and 9l have exhibited good lipoxygenase inhibitory activity. Remaining compounds showed moderate inhibition. The IC50 values of the standard and test samples are summarized in Table 4.

In the Indirect haemolytic assay, 9c and 9g significantly inhibited percent of haemolysis, compounds 9e, 9j, 9k and 9l showed good inhibition of haemolytic process which was caused by the secretary-PLA2 enzyme.
Table 4. IC$_{50}$ values of 9a-1 for anti-inflammatory activity

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ values in µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipoygenase inhibition assay</td>
</tr>
<tr>
<td>9a</td>
<td>1.55</td>
</tr>
<tr>
<td>9b</td>
<td>0.66</td>
</tr>
<tr>
<td>9c</td>
<td>0.40</td>
</tr>
<tr>
<td>9d</td>
<td>0.99</td>
</tr>
<tr>
<td>9e</td>
<td>0.44</td>
</tr>
<tr>
<td>9f</td>
<td>2.0</td>
</tr>
<tr>
<td>9g</td>
<td>0.56</td>
</tr>
<tr>
<td>9h</td>
<td>0.45</td>
</tr>
<tr>
<td>9i</td>
<td>2.33</td>
</tr>
<tr>
<td>9j</td>
<td>0.59</td>
</tr>
<tr>
<td>9k</td>
<td>0.60</td>
</tr>
<tr>
<td>9l</td>
<td>0.65</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.33</td>
</tr>
<tr>
<td>Aristolochic acid</td>
<td>-</td>
</tr>
</tbody>
</table>

“-” = Not determined

2.5 Conclusion

In summary, a series of new benzimidazole derivatives has been synthesized in good yields and screened for antibacterial, antioxidant and anti-inflammatory activities. Compounds 9a, 9c and 9f showed prominent antibacterial activity against both Gram positive and Gram negative bacteria, probably the potent activity may due to electron releasing methyl and methoxy groups. While, rest of the compounds containing electron withdrawing substituents showed moderate activity. Benzimidazole derivatives with alkyl substituents also showed moderate activity probably due to more hydrophobicity of alkyl
groups. Compounds 9e, 9g, 9j and 9k showed good antioxidant activity even in the absence of phenolic groups probably due to divalent sulfur atom in the molecules. More precisely, derivatives with dichloro and methyl group on phenyl ring and propyl and butyl substituents showed good antioxidant activity. Benzimidazoles 9e and 9h bearing dichloro and nitro groups showed good anti-inflammatory activity in lipoxygenase inhibition assay. Also, 9c and 9g carrying methyl group at 3 and 4-positions of phenyl group exhibited good anti-inflammatory activity in indirect hemolytic assay.
References


Appendices
$^1$H NMR Spectrum of 9a

$^{13}$C NMR Spectrum of 9a
$^1$H NMR Spectrum of 9b

$^{13}$C NMR Spectrum of 9b
$^1$H NMR Spectrum of 9c

$^{13}$C NMR Spectrum of 9c
Mass Spectrum of 9a

Mass Spectrum of 9b

Mass Spectrum of 9c