Synthesis, Characterization and Biological evaluation of 8-aryloxy-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidin-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4,5]dec-3-en-2-one
Chapter Abstract

A series of piperidine conjugated benzisoxazole 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, super oxide 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 compounds revealed good activity.

4.1 Introduction

Benzisoxazole scaffold presents a large number of pharmaceutical products, and exhibit antimicrobial, anticonvulsant, antitumor, antipsychotic, antithrombotic, analgesic activities. They have also exhibited antiglycating and cholinesterase-inhibiting properties. Previously we have investigated various biological activities of these benzisoxazole derivatives as antimicrobial and cholinesterase-inhibiting agents. In this
chapter, the antibacterial, antioxidant and anti-inflammatory activities of piperidyl spirolactone linked benzisoxazole derivatives are examined.

4.2 Synthesis of benzisoxazole derivatives

Sodium methoxide induced cyclocondensation of tert-butyl 4-oxopiperidine-1-carboxylate (1) and dimethyl 2-methylenesuccinate (2) in THF to afford 8-tert-butyl 4-methyl 3-methyl-2-oxo-1-oxa-8-azaspiro[4.5]dec-3-ene-4,8-dicarboxylate (3) in 70% yield (via formation of β-hydroxy ester which subsequently undergoes intramolecular cyclisation to form lactone with exocyclic double bond). Later, base induced migration of double bond into the ring gives compound 3. Selective hydrolysis of methyl ester group in compound 3 by lithium hydroxide in methonolic water gets 8-(tert-butoxycarbonyl)-3-methyl-2-oxo-1-oxa-8-azaspiro[4.5]dec-3-ene-4-carboxylic acid (4) in 89% yield. Coupling of compound 4 with 6-fluoro-3-(piperidin-4-yl)benzo[d]isoxazole hydrochloride (5) in presence of EDC·HCl/HOBt in dichloromethane furnishes tert-butyl 4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-2-oxo-1-oxa-8-azaspiro[4.5] dec-3-ene-8-carboxylate (6). Cleavage of tert-butyl oxy group in compound 6 by hydrochloric acid in ether gives 4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one hydrochloride (7). Acylation of compound 7 with various benzoyl chloride derivatives 8 affords final products 8-acyl-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5] dec-3-en-2-one (9). The structures of the synthesized compounds are established with the help of spectral data.
Reagents and reaction conditions: (a) MeONa/THF, 0°C-RT, 8 h. (b) LiOH/MeOH/H\textsubscript{2}O, 0°C-RT, 3 h. (c) 6-fluoro-3-(piperidin-4-yl)benzo[d]isoxazole hydrochloride 5, EDC.HCl/HOBt/DIPEA/CH\textsubscript{2}Cl\textsubscript{2}, 0 °C-RT, 8h. (d) HCl/ether, 0 °C-RT, 1h. (e) 8a-j, TEA/EDC, 0 °C-RT, 3-4 h.

Scheme 1

Table 1. Derivatives of benzisoxazole.

<table>
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<th>Entry</th>
<th>8a-j</th>
<th>9a-j</th>
<th>R</th>
<th>Yield (%)</th>
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<tr>
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<tr>
<td>2</td>
<td>8b</td>
<td>9b</td>
<td>4-C(Me)\textsubscript{3}C\textsubscript{6}H\textsubscript{4}</td>
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</tr>
<tr>
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<td>9c</td>
<td>2,4-Cl\textsubscript{2}C\textsubscript{6}H\textsubscript{3}</td>
<td>72</td>
</tr>
</tbody>
</table>
4. **8d**

5. **8e**

6. **8f**

7. **8g**

8. **8h**

9. **8i**

10. **8j**

- **9d** 3-BrC₆H₄ 83
- **9e** 3,5-(NO₂)₂C₆H₃ 65
- **9f** 3,4,5-(MeO)₃C₆H₂ 70
- **9g** 3-NO₂C₆H₄ 68
- **9h** 4-MeOC₆H₄ 72
- **9i** 2,6-F₂C₆H₃ 60
- **9j** 3-ClC₆H₄ 73
4.3 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 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.

4.3.1 General procedure for the synthesis of 8-tert-butyl-4-methyl-3-methyl-2-oxo-1-oxa-8-azaspiro[4,5]dec-3-ene-4,8-dicarboxylate (3): To a solution of tert-butyl 4-oxopiperidine-1-carboxylate (20 mmol) and dimethyl 2-methylenesuccinate (20 mmol) in THF (50 mL), a solution of sodium methoxide (40 mmol) was added at 0°C. The reaction mixture was stirred at room temperature for 8h. After completion of the reaction, 10 mL of water were added; the organic layer was extracted with ethyl acetate and distilled under reduced pressure to get product 3 in good yield.

Viscous liquid; IR (KBr) cm$^{-1}$: 1555 (Olefin C=C str.), 1740 (Ester CO str.), 3045 (Aromatic CH str.); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 3.77 (s, 3H, OMe), 3.30-3.41 (m, 4H, CH$_2$), 2.43 (s, 3H, CH$_3$), 1.65-1.80 (m, 4H, CH$_2$), 1.38 (s, 9H, (CH$_3$)$_3$); MS (ESI): m/z 326 (M+1); Anal. calcd. for C$_{16}$H$_{23}$NO$_6$: C, 59.06; H, 7.13; N, 4.31. Found: C, 59.15; H, 7.21; N, 4.36.

4.3.2 General procedure for the synthesis of 8-(tert-butoxycarbonyl)-3-methyl-2-oxo-1-oxa-8-azaspiro[4,5]dec-3-ene-4-carboxylic acid (4): To a solution of compound
3 (20 mmol) in methanol 30 mL, LiOH (20 mmol) in water (30 mL) was added at 0 °C and stirred for 3 h at room temperature. After completion of the reaction, the mixture was concentrated under reduced pressure and the residue was extracted with ethyl acetate (3 x 50 mL), the solvent was removed under reduced pressure to get product 4.

White solid; mp. 126-128 °C; IR (KBr) cm⁻¹: 1715 (Acid CO str.), 1742 (Ester CO str.), 3042 (Aromatic CH str.), 3215 (Acid OH str.); ¹H NMR (400 MHz, CDCl₃) δ 10.50 (s, 1H, COOH), 3.30-3.40 (m, 4H, CH₂), 2.43 (s, 3H, CH₃), 1.65-1.80 (m, 4H, CH₂), 1.38 (s, 9H, (CH₃)₃). MS (ESI): m/z 311 (M+1); Anal. calcd. for C₁₅H₂₁NO₆: C, 57.87; H, 6.80; N, 4.50. Found: C, 57.96; H, 6.88; N, 4.58.

4.3.3 General procedure for the synthesis of tert-butyl 4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-2-oxo-1-oxa-8-azaspiro[4.5]dec-3-ene-8-carboxylate (6): To a solution of compound 4 (20 mmol) and 6-fluoro-3-(piperidin-4-yl)benzo[d]isoxazole hydrochloride¹⁴ 5 (20 mmol) in dichloromethane (40 mL); 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl) (20 mmol) and Hydroxybenzotriazole (HOBt) (2 mmol) were added at 0°C and the reaction mixture was stirred at room temperature for 8h. After completion of the reaction, 20 mL of water were added; the organic layer was extracted with ethyl acetate and distilled under reduced pressure to get product 6 in good yield.

White solid; mp. 130-132 °C; IR (KBr) cm⁻¹:1660 (Amide CO str.), 1742 (Ester CO str.), 3039 (Aromatic CH str.). ¹H NMR (400 MHz, CDCl₃) δ 7.54 (d, J=7.8 Hz, 1H, Ar-H), 7.24 (d, J=7.8 Hz, 1H, Ar-H), 6.97 (s, 1H, Ar-H), 3.30-3.41 (m, 8H, CH₂), 2.78 (m, 1H, CH), 2.42 (s, 3H, CH₃), 1.70-1.86 (m, 8H, CH₂), 1.38 (s, 9H, (CH₃)₃). MS (ESI): m/z 513
(M+1); Anal. calcd. for C_{27}H_{32}FN_{3}O_{6}: C, 63.15; H, 6.28; N, 8.18. Found: C, 63.19; H, 6.35; N, 8.26.

4.3.4 General procedure for the synthesis of 4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one hydrochloride (7): To a solution of compound 6 (20 mmol) in diethyl ether (40 mL), a saturated solution of HCl in ether was added at 0°C and stirred for 1h. The reaction mixture was concentrated under reduced pressure to get compound 7 in high yield. White solid; mp. 180-182 °C. IR (KBr) cm⁻¹: 1664 (Amide CO str.), 1744 (Ester CO str.), 3035 (Aromatic CH str.), 3320 (Amine NH Str.). \(^1\)H NMR (400 MHz, DMSO-d₆) δ 7.56 (d, \(J=7.8\) Hz, 1H, Ar-H), 7.24 (d, \(J=7.8\) Hz, 1H, Ar-H), 6.97 (s, 1H, Ar-H), 6.51 (s, 2H, NH₂), 3.3-3.42 (m, 8H, CH₂), 2.78 (m, 1H, CH), 2.51 (s, 3H, CH₃), 2.15-2.30 (m, 4H, CH₂), 1.70-1.90 (m, 4H, Ar-H). MS (ESI): m/z 450 (M+1). Anal. calcd. for C_{22}H_{25}ClFN_{3}O_{4}: C, 58.73; H, 5.60; N, 9.34. Found: C, 58.79; H, 5.68; N, 9.42.

4.3.5. General procedure for the synthesis of 8-acyl-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9): To a solution of compound 7 (5 mmol) and triethyl amine (5 mmol) in dichloromethane (20 mL); acyl chloride (8) (5 mmol) was added at 0°C and stirred at room temperature for 3-4 h. After the completion of the reaction, 20 mL of water were added and extracted the reaction mixture with dichloromethane (2 x 20 mL). The organic layer was concentrated under reduced pressure to get products 9 (a-j) (Table 1) which were purified by column chromatography using CHCl₃:MeOH (9:1, v:v) as eluent.

8-(4-Chlorobenzoyl)-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9a): White solid; mp. 108-110°C.
IR (KBr) cm⁻¹: 1660 (Amide CO str.), 1740 (Ester CO str.), 3045 (Aromatic CH str.). ¹H NMR (400 MHz, CDCl₃, δ, ppm): 8.08 (d, J = 7.8 Hz, 2H, Ar-H), 7.75 (d, J = 8.2 Hz, 2H, Ar-H), 7.61 (d, J = 7.2 Hz, 1H, Ar-H), 7.29 (d, J = 7.2 Hz, 1H, Ar-H), 7.03 (s, 1H, Ar-H), 3.29-3.39 (m, 8H, CH₂), 2.72 (m, 1H, CH), 2.52 (s, 3H, CH₃), 1.60-1.90 (m, 8H, CH₂).

MS (ESI): m/z 553 (M+1). Anal. calcd. for C₂₉H₂₇ClFN₃O₅: C, 63.10; H, 4.93; N, 7.61. Found: C, 63.15; H, 4.96; N, 7.63.

8-(4-(tert-Butyl)benzoyl)-4-(4-(6-fluorobenz[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9b):

White solid; mp. 108-110°C. IR (KBr) cm⁻¹: 1671 (Amide CO str.), 1749 (Ester CO str.), 3039 (Aromatic CH str.); ¹H NMR (400 MHz, CDCl₃) δ 7.95 (d, J = 7.8 Hz, 2H, Ar-H), 7.55 (d, J = 7.2 Hz, 1H, Ar-H), 7.47 (d, J = 7.8 Hz, 2H, Ar-H), 7.24 (d, J = 7.2 Hz, 1H, Ar-H), 6.97 (s, 1H, Ar-H), 3.28-3.36 (m, 8H, CH₂), 2.71 (m, 1H, CH), 2.53 (s, 3H, CH₃),
1.60-1.91 (m, 8H, CH₂), 1.35 (s, 9H, CMe₃). MS (ESI): m/z 574 (M+1). Anal. calcd. for C₃₃H₃₆FN₃O₅: C, 69.09; H, 6.33; N, 7.32. Found: C, 69.12; H, 6.39; N, 7.36.

8-(2,4-Dichlorobenzoyl)-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9c):

White solid; mp. 114-116°C. IR (KBr) cm⁻¹: 1655 (Amide CO str.), 1756 (Ester CO str.), 3049 (Aromatic CH str.); ¹H NMR (400 MHz, CDCl₃) δ 7.75 (s, 1H, Ar-H), 7.49-7.55 (m, 3H, Ar-H), 7.26 (d, J = 7.8 Hz, 1H, Ar-H), 7.02 (s, 1H, Ar-H), 3.27–3.35 (m, 8H, CH₂), 2.72 (m, 1H, CH), 2.50 (s, 3H, CH₃), 1.62–1.88 (m, 8H, CH₂); MS (ESI): m/z 587 (M+1); Anal. calcd. for C₂₉H₂₆Cl₂FN₅O₅: C, 59.39; H, 4.47; N, 7.17. Found: C, 59.45; H, 4.52; N, 7.25.

8-(3-Bromobenzoyl)-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9d):
White solid; mp. 128-130°C; IR (KBr) cm\(^{-1}\): 1659 (Amide CO str.), 1765 (Ester CO str.), 3055 (Aromatic CH str.); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.25 (s, 1H, Ar-H), 8.05 (d, \(J = 7.8\) Hz, 1H, Ar-H), 7.98 (d, \(J = 7.6\) Hz, 1H, Ar-H), 7.55 (m, 2H, Ar-H), 7.26 (m, 1H, Ar-H), 7.05 (s, 1H, Ar-H), 3.25-3.35 (m, 8H, CH\(_2\)), 2.70 (m, 1H, CH), 2.52 (s, 3H, CH\(_3\)), 1.60-1.84 (m, 8H, CH\(_2\)); MS (ESI): m/z 597 (M+1); Anal. calcd. for C\(_{29}\)H\(_{27}\)BrFN\(_3\)O\(_5\): C, 58.40; H, 4.56; N, 7.05. Found: C, 58.46; H, 4.60; N, 7.11.

8-(3,5-Dinitrobenzoyl)-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9e):

White solid; mp. 184-186°C; IR (KBr) cm\(^{-1}\): 1665 (Amide CO str.), 1771 (Ester CO str.), 3032 (Aromatic CH str.); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 9.01 (d, \(J = 2.8\) Hz, 2H, Ar-H), 8.93 (s, 1H, Ar-H), 7.59 (d, \(J = 7.2\) Hz, 1H, Ar-H), 7.32 (d, \(J = 7.2\) Hz, 1H, Ar-H), 7.01 (s, 1H, Ar-H), 3.28-3.39 (m, 8H, CH\(_2\)), 2.73 (m, 1H, CH), 2.49 (s, 3H, CH\(_3\)), 1.62-1.81 (m, 8H, CH\(_2\)); Anal. calcd. for C\(_{29}\)H\(_{26}\)FN\(_5\)O\(_9\): C, 57.33; H, 4.31; N, 11.53. Found: C, 57.39; H, 4.36; N, 11.59. MS (ESI): m/z 608 (M+1).
4-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-8-(3,4,5-
trimethoxy benzoyl)-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9f):

White solid; mp. 188-190°C; IR (KBr) cm\(^{-1}\): 1660 (Amide CO str.), 1765 (Ester CO str.),
3073 (Aromatic CH str.); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.55 (d, \(J = 7.2\) Hz, 1H, Ar-H),
7.24 (d, \(J = 7.4\) Hz, 1H, Ar-H), 7.17 (s, 2H, Ar-H), 6.97 (s, 1H, Ar-H), 3.83 (s, 6H, OMe),
3.81 (s, 3H, OMe), 3.25-3.39 (m, 8H, CH\(_2\)), 2.72 (m, 1H, CH), 2.50 (s, 3H, CH\(_3\)), 1.61-
1.80 (m, 8H, CH\(_2\)); MS (ESI): m/z 608 (M+1); Anal. calcd. for C\(_{32}\)H\(_{34}\)FN\(_3\)O\(_8\): C, 63.25;
H, 5.64; N, 6.92. Found: C, 63.30; H, 5.71; N, 6.97.

4-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-8-(3-nitro
benzoyl)-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9g):
White solid; mp. 194-196°C; IR (KBr) cm⁻¹: 1669 (Amide CO str.), 1771 (Ester CO str.), 3061 (Aromatic CH str.); ¹H NMR (400 MHz, CDCl₃) δ 8.72 (s, 1H, Ar-H), 8.51 (d, J = 8.0 Hz, 1H, Ar-H), 8.42 (d, J = 8.0 Hz, 1H, Ar-H), 7.89 (t, J = 7.8 Hz, 1H, Ar-H), 7.55 (d, J = 7.2 Hz, 1H, Ar-H), 7.24 (d, J = 7.3 Hz, 1H, Ar-H), 6.97 (s, 1H, Ar-H), 3.29-3.42 (m, 8H, CH₂), 2.68 (m, 1H, CH), 2.48 (s, 3H, CH₃), 1.65-1.82 (m, 8H, CH₂). MS (ESI): m/z 563 (M+1); Anal. calcd. for C₂₉H₂₇FN₄O₇: C, 61.92; H, 4.84; N, 9.96. Found: C, 61.98; H, 4.89; N, 9.98.

4-(4-(6-Fluorobenz[d]isoxazol-3-yl)piperidine-1-carbonyl)-8-(4-methoxybenzoyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9h):

White solid; mp. 108-110 °C. IR (KBr) cm⁻¹: 1659 (Amide CO str.), 1782 (Ester CO str.), 3047 (Aromatic CH str.); ¹H NMR (400 MHz, CDCl₃): δ 7.92 (d, J = 8.0 Hz, 2H, Ar-H), 7.55 (d, J = 7.6 Hz, 1H, Ar-H), 7.26 (d, J=7.6 Hz, 1H, Ar-H), 7.16 (d, J=8.0 Hz, 2H, Ar-H), 6.97 (s, 1H, Ar-H), 3.82 (s, 1H, OMe), 3.32-3.45 (m, 8H, CH₂), 2.70 (m, 1H, CH), 2.49 (s, 3H, CH₃), 1.62-1.85 (m, 8H, CH₂). MS (ESI): m/z: 548 (M+1); Anal. calcd. for C₃₀H₃₀FN₅O₆: C, 65.80; H, 5.52; N, 7.67. Found: C, 65.85; H, 5.59; N, 7.73.
8-(2,6-Difluorobenzoyl)-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9i):

White solid; mp. 128-130 °C; IR (KBr) cm⁻¹: 1667 (Amide CO str.), 1774 (Ester CO str.), 3059 (Aromatic CH str.); ¹H NMR (400 MHz, CDCl₃) δ 7.53-7.56 (m, 2H, Ar-H), 7.19-7.24 (m, 3H, Ar-H), 6.97 (s, 1H, Ar-H), 3.30-3.44 (m, 8H, CH₂), 2.71 (m, 1H, CH), 2.54 (s, 3H, CH₃), 1.60-1.85 (m, 8H, CH₂). MS (ESI): m/z 554 (M+1); Anal. calcd. for C₂₉H₂₆F₃N₃O₅: C, 62.93; H, 4.73; N, 7.59. Found: C, 62.96; H, 4.79; N, 7.64.

8-(3-Chlorobenzoyl)-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (9j):

White solid; mp. 110-112°C; IR (KBr) cm⁻¹: 1665 (Amide CO str.), 1755 (Ester CO str.), 3068 (Aromatic CH str.); ¹H NMR (400 MHz, CDCl₃): δ 8.22 (s, 1H, Ar-H), 8.03 (d, J = 7.8 Hz, 1H, Ar-H), 7.96 (d, J = 7.6 Hz, 1H, Ar-H), 7.52 (m, 2H, Ar-H), 7.23 (m, 1H, Ar-H), 7.02 (s, 1H, Ar-H), 3.26-3.36 (m, 8H, CH₂), 2.71 (m, 1H, CH), 2.53 (s, 3H, CH₃),
1.61-1.83 (m, 8H, CH\(_2\)); MS (ESI): m/z 553 (M+1); Anal. calcd. for C\(_{29}\)H\(_{27}\)ClFN\(_3\)O\(_5\): C, 63.10; H, 4.93; N, 7.61. Found: C, 63.10; H, 4.93; N, 7.61.

4.4 Biological activity

4.4.1 Antibacterial Activity

Disc diffusion method (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 disc (i.e., zone of inhibition). The presence of a clear zone of inhibition surrounding the disc is indicative of inhibitory (antimicrobial) activity against the organism.

All newly synthesized compounds were evaluated for their antibacterial activity against 5 human pathogenic bacterial strains. They 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 disc 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\(^\circ\)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. The discs (6 mm diameter) were impregnated with 5 mg and 10 mg/mL of each compound and placed on the
inoculated nutrient agar. Then, the inoculated plates were incubated at 37±0.1°C for 24 h. Gentamicin and Chloramphenicol are used as positive controls.

Antibacterial activity was evaluated by measuring the zone of inhibition against each test organism and the results are summarized in Table 2.

4.4.1.1 Results and Discussion

The zone of inhibition of the compounds indicated that **9a-b** and **9d-g** showed good antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Shigella flexneri* and *Bacillus subtilis*. While the compounds **9c**, **9h**, **9i** and **9j** showed moderate antibacterial activity. Compound with dinitro substituent showed highest antibacterial activity. Most of the compounds exhibited antibacterial activity probably due to the presence of bioactive benzisoxazole moiety.

**Table 2. Antibacterial activity of benzisoxazoles 9a-j.**

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<th>Compound</th>
<th><em>Escherichia coli</em></th>
<th><em>Bacillus subtilis</em></th>
<th><em>Klebsiella pneumoniae</em></th>
<th><em>Salmonella typhi</em></th>
<th><em>Shigella flexneri</em></th>
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<tr>
<td><strong>9d</strong></td>
<td>10</td>
<td>13</td>
<td>9</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td><strong>9e</strong></td>
<td>16</td>
<td>15</td>
<td>16</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td><strong>9f</strong></td>
<td>10</td>
<td>15</td>
<td>11</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td><strong>9g</strong></td>
<td>11</td>
<td>12</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td><strong>9h</strong></td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>9i</strong></td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>9j</strong></td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

* Inhibition zones including cup borer (6.0 mm) diameter

Positive control zone is 35 to 40 mm

“-“ = Not active.
4.4.2 Antioxidant activity

Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and 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 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.

4.4.2.1 DPPH radical scavenging assay

DPPH radical scavenging assays were performed in 300 µL reaction mixtures containing 200 µL of 0.1 mM DPPH-ethanol solution, 90 µL of 50 mM Tris-HCl buffer (pH = 7.4), and 10 µL of deionised water (as control) and various concentrations of compounds 9a-j (1.8-9.0 µM). Ascorbic acid was used as a 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 percent radical scavenging activity was calculated according to the following equation,

\[
\text{Inhibition (\%)} = \left( \frac{\text{Absorbance control} - \text{Absorbance Sample}}{\text{Absorbance Control}} \right) \times 100.
\]

The DPPH radical scavenging activity is demonstrated in Figure 1 and Table 3.
4.2.2 Hydroxy l radical scavenging assay

The reaction mixture in final volume of 2 mL containing 0.1 mL of EDTA (1 mM), 0.01 mL of FeCl₃ (10 mM), 0.1 mL of H₂O₂ (10 mM), 0.36 mL of deoxyribose (10 mM), 1 mL of compounds 9a-j (concentrations from 1.8-9.0 µM), 0.33 mL of phosphate buffer (50 mM, pH = 7.4) and 0.1 mL ascorbic acid (1 mM) were added in sequence. The mixture was incubated at 37°C for 1h. 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°C for 20 min to develop a pink chromogen which was measured at 532 nm. Ascorbic acid was used as a positive control. Percent inhibition was evaluated by using the following equation.

\[
\text{Inhibition} \, (\%) = \left( \frac{\text{Absorbance control} - \text{Absorbance Sample}}{\text{Absorbance Control}} \right) \times 100
\]

The potency of benzisoxazoles for hydroxyl radical scavenging activity is illustrated in Figure 2 and Table 3.
4.4.2.3 Superoxide anion radical scavenging assay

One mL of NBT (156 µM NBT in 100 mM phosphate buffer of pH = 7.4), 1 mL of NADH (468 µM in 100 mM phosphate buffer of pH = 7.4) and varying concentrations of compounds 9a-j (1.8-9.0 µM) 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 and it is illustrated in Figure 3 and Table 3.
Figure 3. Superoxide radical scavenging assay.

4.4.2.4 Results and Discussion

In all the antioxidant assays compounds 9b, 9f and 9h containing electron donating groups exhibited good inhibitory activity. The remaining compounds showed moderate antioxidant activity. At this stage, it is not possible to give any conclusive explanation for the antioxidant activities of benzisoxazole derivatives even in the absence of essential phenolic group.

Table 3. Antioxidant activity of benzisoxazoles 9a-j.

<table>
<thead>
<tr>
<th>Compound</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>7.0</td>
<td>7.5</td>
<td>7.3</td>
</tr>
<tr>
<td>9b</td>
<td>4.1</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>9c</td>
<td>6.2</td>
<td>6.4</td>
<td>6.5</td>
</tr>
<tr>
<td>9d</td>
<td>7.9</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>9e</td>
<td>6.5</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>9f</td>
<td>4.9</td>
<td>4.6</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>9g</td>
<td>9h</td>
<td>9i</td>
</tr>
<tr>
<td>-----</td>
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<td>----</td>
</tr>
<tr>
<td>Ass.</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Ac.</td>
<td>5.2</td>
<td>5.1</td>
<td>4.7</td>
</tr>
<tr>
<td>9j</td>
<td>8.5</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Ac.</td>
<td>7.8</td>
<td>3.5</td>
<td>3.4</td>
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<tr>
<td>Quercetin</td>
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<td>-</td>
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</table>

“-” = Not active

4.4.3 Anti-inflammatory activity:

4.4.3.1 Lipoxygenase inhibition assay

Lipoxygenase inhibition assay\(^{18}\) was carried out using linoleic acid as substrate and with lipoxgenase enzyme. To a solution of 0.1 mL of 0.2 M borate buffer (pH = 9.0), containing 0.1 mL of 1000 units lipoxidase enzyme varying concentrations of compounds 9a-j (1.8-9 µM) were added and incubated. The tubes were agitated and incubated at room temperature 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 a reference standard drug. Percent (%) inhibition was calculated by the equation,

\[
\text{Inhibition (\%)} = \left(\frac{\text{Absorbance control} - \text{Absorbance Sample}}{\text{Absorbance Control}}\right) \times 100
\]

Lipoxygenase inhibition activity of benzisoxazoles is summarized in Figure 4 and Table 4.
Figure 4. Lipoxygenase inhibition assay.

4.4.3.2 Inhibition of PLA₂ induced haemolysis in human erythrocytes

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 haemoglobin was read at 540 nm. For inhibition studies 10 μg of venom sample (secretory-PLA₂ purchased from sigma) were incubated with various concentrations of compounds 9a-j (17 -90μM 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 and inhibitory activities are summarized in Figure 5 and Table 4.
The results of anti-inflammatory activity are summarized in Table 4. In both lipooxygenase inhibition and phospholipase A2 inhibition assays compounds bearing electron withdrawing groups 9e and 9g exhibited good anti-inflammatory activity, the remaining compounds showed moderate activity probably due to the absence of deactivating groups on phenyl ring. It is interesting to note that the compounds bearing activating groups on phenyl ring showed good antioxidant activity, whereas those with deactivating groups exhibited anti-inflammatory activities.
Table 4. Anti-inflammatory activity of benzisoxazoles 9a-j.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ values in µM</th>
<th>Lipoygenase inhibition assay</th>
<th>PLA$_2$ inhibition assay</th>
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</thead>
<tbody>
<tr>
<td>9a</td>
<td>8.6</td>
<td>79.0</td>
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</tr>
<tr>
<td>9b</td>
<td>4.1</td>
<td>37.4</td>
<td></td>
</tr>
<tr>
<td>9c</td>
<td>6.2</td>
<td>62.7</td>
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</tr>
<tr>
<td>9d</td>
<td>7.9</td>
<td>76.9</td>
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</tr>
<tr>
<td>9e</td>
<td>3.9</td>
<td>40.3</td>
<td></td>
</tr>
<tr>
<td>9f</td>
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<td>9g</td>
<td>3.9</td>
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<td>9h</td>
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<tr>
<td>9i</td>
<td>8.5</td>
<td>81.1</td>
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<tr>
<td>9j</td>
<td>7.4</td>
<td>75.3</td>
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<tr>
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<tr>
<td>Aristolochic acid</td>
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<td>30.0</td>
<td></td>
</tr>
</tbody>
</table>

“-” = Not determined

4.5 Conclusion

In summary, a series of new benzisoxazole derivatives have been synthesized in good yields and screened for antibacterial, antioxidan and anti-inflammatory activities.

Compounds 9a-b and 9d-g showed good antibacterial activity against *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi*, *Shigella flexneri* and *Bacillus subtilis*. Benzisoxazoles 9b, 9f and 9h bearing electron donating groups exhibited prominent antioxidant activity and 9e and 9g showed good anti-inflammatory activity.
References


Appendices
\(^1\)H NMR Spectrum of 9a

Mass spectrum of 9a
$^1$H NMR Spectrum of 9b

Mass spectrum of 9b
Section 4

$^1$H NMR Spectrum of $9c$

Mass spectrum of $9c$