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New synthetic benzisoxazole derivatives as antimicrobial, antioxidant and anti-inflammatory agents

Chalya Mallappaji Shivaprasad, Swamy Jagadish, Toreshettahally Ramesh Swaroop, Chakrabhavi Dhananjaya Mohan, Rangaswamy Roopashree, Kothanahally Shivaramu Sharath Kumar and Kanchugarakoppal Subbegowda Rangappa *

Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore 570 006, India

** Corresponding author at: Department of Studies in Chemistry, University of Mysore, Manasagangotri, Mysore 570 006, India. Tel.: +91.0821.2419661; fax: +91.0821.2500846. E-mail address: rangappaks@gmail.com (K.S. Rangappa).*

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ABSTRACT

A series of piperidine conjugated benzisoxazole derivatives were synthesized and evaluated for their antibacterial, anti-oxidant and anti-inflammatory activities. The results showed that most of the tested compounds exhibit 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 anti-oxidant 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.

1. Introduction

Benzisoxazole scaffold present in large number of pharmaceutical products with antimicrobial [1], anticonvulsant [2,3], antitumor [4,5], antipsychotic [6-8], antithrombotic [9], analgesic activities [10]. They have also exhibited antiglycating [11] and cholinesterase-inhibiting properties [12,13]. Previously we have investigated various biological activities of these benzisoxazole derivatives as antimicrobial [1] and cholinesterase-inhibiting agents [13]. In continuation of this work, we report herein antibacterial, antioxidant and anti-inflammatory activities of piperidyl spirolactone linked benzisoxazole derivatives.

2. Experimental

2.1. Instrumentation

The melting points were determined on Selaco melting point apparatus and are uncorrected. Infrared spectra were recorded on Shimadzu FT-IR model 8300 spectrometer. ^{1}H NMR spectra were recorded on an NMR spectrometer operating at 400 MHz 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 60F_{254}) using UV light for visualization. All chemicals were obtained from Aldrich, Fluka and Merck Chemicals.

2.2. Synthesis

2.2.1. 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 8 h. After completion of the reaction, 10 mL of water was added; the organic layer was extracted with ethyl acetate distilled under reduced pressure to get product **3** in good yield (Scheme 1). Colour: White. Yield: 70% (4.55 g). Viscous liquid. FT-IR (KBr, cm^{-1}): 1555 (Olefin C=C str.), 1740 (Ester CO str.), 3045 (Aromatic CH str.). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 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, $(CH_3)_3$). 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%. MS (*m/z*): 326 (M+1).

2.2.2. General procedure for the synthesis of 8-(tert-butoxy carbonyl)-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 and 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, reaction mass 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** (Scheme 1). Colour: White. Yield: 89% (5.53 g). M.p.: 126-128 °C. FT-IR (KBr, cm^{-1}): 1715 (Acid CO str.), 1742 (Ester CO str.), 3042 (Aromatic CH str.), 3215 (Acid OH str.).

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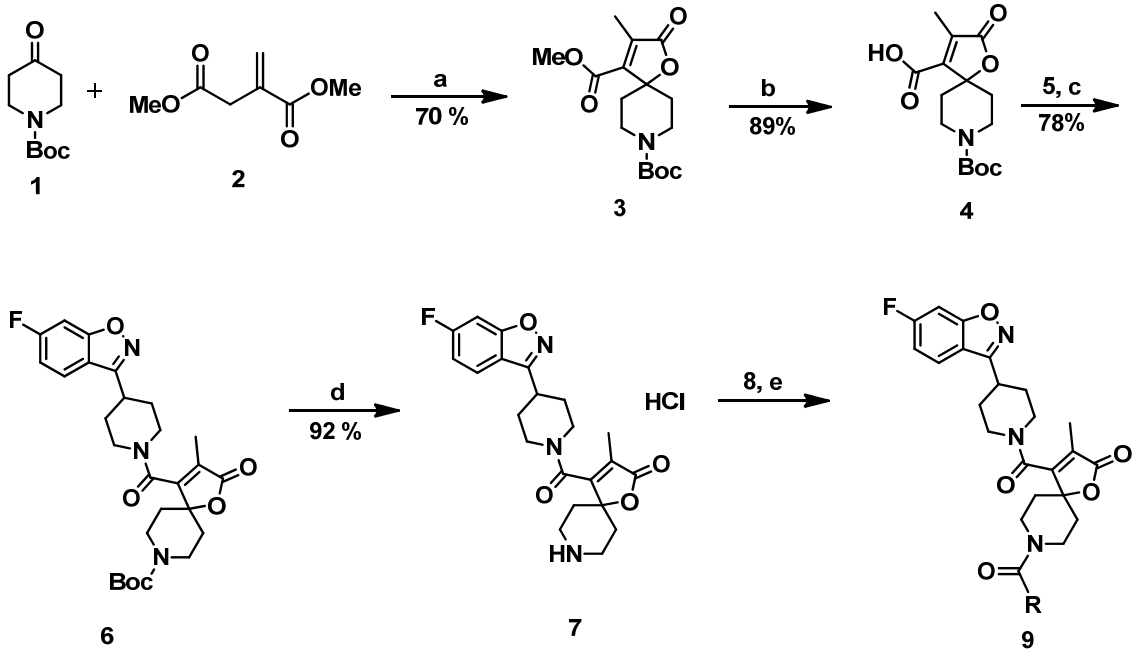

Reagents and reaction conditions: (a) MeONa/THF, 0 °C-RT, 8 h. (b) LiOH/MeOH/H_2O, 0 °C-RT, 3 h. (c) 6-fluoro-3-(piperidin-4-yl)benzo[d]isoxazole hydrochloride **5**, EDC·HCl/HOBt/DIPEA/CH_2Cl_2, 0 °C-RT, 8h. (d) HCl/ether, 0 °C-RT, 1h. (e) RCOCl **8**, TEA/EDC, 0 °C-RT, 3-4 h.

Scheme 1

^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 10.5 (s, 1H, COOH), 3.30-3.40 (m, 4H, CH_2), 2.43 (s, 3H, CH_3), 1.65-1.80 (m, 4H, CH_2), 1.38 (s, 9H, $(CH_3)_3$). Anal. calcd. for $C_{15}H_{21}NO_6$: C, 57.87; H, 6.80; N, 4.50. Found: C, 57.96; H, 6.88; N, 4.58. MS (*m/z*): 311 (M+1).

2.2.3. 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 hydrochloride [14] **5** (20 mmol) in dichloromethane (40 mL); 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC·HCl) (20 mmol) and hydroxybenzotriazole (HOBt) (2 mmol) was added at 0 °C and the reaction mixture was stirred at room temperature for 8 h. After completion of the reaction, 20 mL of water was added; the organic layer was extracted with ethyl acetate and distilled under reduced pressure to get product **6** in good yield (Scheme 1). Colour: White. Yield: 78% (8.00 g). M.p.: 130-132 °C. FT-IR (KBr, cm^{-1}): 1660 (Amide CO str.), 1742 (Ester CO str.), 3039 (Aromatic CH str.). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 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, CH_2), 2.78 (m, 1H, CH), 2.42 (s, 3H, CH_3), 1.70-1.86 (m, 8H, CH_2), 1.38 (s, 9H, $(CH_3)_3$). Anal. calcd. for $C_{27}H_{32}FN_3O_6$: C, 63.15; H, 6.28; N, 8.18. Found: C, 63.19; H, 6.35; N, 8.26. MS (*m/z*): 513 (M+1).

2.2.4. General procedure for the synthesis of 4-(4-(6-fluoro benzo[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 for 1 h. The reaction mixture was concentrated under reduced pressure to get compound **7** in high yield (Scheme 1). Colour: White. Yield: 92% (8.26 g). M.p.: 180-182 °C. FT-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-d_6, δ, ppm): 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, 1H, NH), 3.3-3.42 (m, 8H, CH_2), 2.78 (m, 1H, CH), 2.51 (s, 3H, CH_3), 2.15-2.30 (m, 4H, CH_2), 1.70-1.90 (m, 4H, CH_2). Anal. calcd. for $C_{22}H_{25}ClFN_3O_4$: C, 58.73; H, 5.60; N, 9.34. Found: C, 58.79; H, 5.68; N, 9.42. MS (*m/z*): 450 (M+1).

2.2.5. 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 was added and extracted the reaction mixture with dichloromethane (2 x 20 mL). The organic layer was concentrated under reduced pressure to get products **9** (Table 1) which were purified by column chromatography using $CHCl_3$:MeOH (9:1, *v*:*v*) as eluent (Scheme 1).

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**): Colour: White. Yield: 81% (2.23 g). M.p.: 108-110 °C. FT-IR (KBr, cm^{-1}): 1660 (Amide CO str.), 1740 (Ester CO str.), 3045 (Aromatic CH str.). ^{1}H NMR (400 MHz, $CDCl_3$, 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, CH_2), 2.72 (m, 1H, CH), 2.52 (s, 3H, CH_3), 1.60-1.90 (m, 8H, CH_2). Anal. calcd. for $C_{29}H_{27}ClFN_3O_5$: C, 63.10; H, 4.93; N, 7.61. Found: C, 63.15; H, 4.96; N, 7.63%. MS (*m/z*): 553 (M+1).

8-(4-(tert-Butyl)benzoyl)-4-(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (**9b**): Colour: White. Yield: 76% (2.17 g). M.p.: 108-110 °C. FT-IR (KBr, cm^{-1}): 1671 (Amide CO str.), 1749 (Ester CO str.), 3039 (Aromatic CH str.). ^{1}H NMR (400 MHz, $CDCl_3$, δ,

ppm): 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, CH_2), 2.71 (m, 1H, CH), 2.53 (s, 3H, CH_3), 1.60-1.91 (m, 8H, CH_2), 1.35 (s, 9H, CMe_3). Anal. calcd. for $C_{33}H_{36}FN_3O_5$: C, 69.09; H, 6.33; N, 7.32. Found: C, 69.12; H, 6.39; N, 7.36%. MS (*m/z*): 574 (M+1).

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**): Colour: White. Yield: 72% (2.10 g). M.p.: 114-116 °C. FT-IR (KBr, cm^{-1}): 1655 (Amide CO str.), 1756 (Ester CO str.), 3049 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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, CH_2), 2.72 (m, 1H, CH), 2.50 (s, 3H, CH_3), 1.62-1.88 (m, 8H, CH_2). Anal. calcd. for $C_{29}H_{26}Cl_2FN_3O_5$: C, 59.39; H, 4.47; N, 7.17. Found: C, 59.45; H, 4.52; N, 7.25%. MS (*m/z*): 587 (M+1).

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**): Colour: White. Yield: 83% (2.47 g). M.p.: 128-130 °C. FT-IR (KBr, cm^{-1}): 1659 (Amide CO str.), 1765 (Ester CO str.), 3055 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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). Anal. calcd. for $C_{29}H_{27}BrFN_3O_5$: C, 58.40; H, 4.56; N, 7.05. Found: C, 58.46; H, 4.60; N, 7.11. MS (*m/z*): 597 (M+1).

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**): Colour: White. Yield: 65% (1.97 g). M.p.: 184-186 °C. FT-IR (KBr, cm^{-1}): 1665 (Amide CO str.), 1771 (Ester CO str.), 3032 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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 $C_{29}H_{26}FN_5O_9$: C, 57.33; H, 4.31; N, 11.53. Found: C, 57.39; H, 4.36; N, 11.59. MS (*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**): Colour: White. Yield: 70% (2.12 g). M.p.: 188-190 °C. FT-IR (KBr, cm^{-1}): 1660 (Amide CO str.), 1765 (Ester CO str.), 3073 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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). Anal. calcd. for $C_{32}H_{34}FN_3O_8$: C, 63.25; H, 5.64; N, 6.92. Found: C, 63.30; H, 5.71; N, 6.97. MS (*m/z*): 608 (M+1).

4-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-8-(3-nitrobenzoyl)-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (**9g**): Colour: White. Yield: 68% (1.91 g). M.p.: 194-196 °C. FT-IR (KBr, cm^{-1}): 1669 (Amide CO str.), 1771 (Ester CO str.), 3061 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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, CH_2), 2.68 (m, 1H, CH), 2.48 (s, 3H, CH_3), 1.65-1.82 (m, 8H, CH_2). Anal. calcd. for $C_{29}H_{27}FN_4O_7$: C, 61.92; H, 4.84; N, 9.96. Found: C, 61.98; H, 4.89; N, 9.98. MS (*m/z*): 563 (M+1).

4-(4-(6-Fluorobenzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-8-(4-methoxybenzoyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (**9h**): Colour: White. Yield: 72% (1.96 g). M.p.: 108-110 °C. FT-IR (KBr, cm^{-1}): 1659 (Amide CO str.), 1782 (Ester CO str.), 3047 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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, 3H, OMe), 3.32-3.45 (m, 8H, CH_2), 2.70 (m, 1H, CH), 2.49 (s, 3H, CH_3), 1.62-1.85 (m, 8H, CH_2). Anal. calcd. for $C_{30}H_{30}FN_3O_6$: C, 65.80; H, 5.52; N, 7.67. Found: C, 65.85; H, 5.59; N, 7.73. MS (*m/z*): 548 (M+1).

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**): Colour: White. Yield: 60% (1.65 g). M.p.: 128-130 °C. FT-IR (KBr, cm^{-1}): 1667 (Amide CO str.), 1774 (Ester CO str.), 3059 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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, CH_2), 2.71 (m, 1H, CH), 2.54 (s, 3H, CH_3), 1.60-1.85 (m, 8H, CH_2). Anal. calcd. for $C_{29}H_{26}F_3N_3O_5$: C, 62.93; H, 4.73; N, 7.59. Found: C, 62.96; H, 4.79; N, 7.64. MS (*m/z*): 554 (M+1).

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**): Colour: White. Yield: 73% (2.01 g). M.p.: 110-112 °C. FT-IR (KBr, cm^{-1}): 1665 (Amide CO str.), 1755 (Ester CO str.), 3068 (Aromatic CH str.). ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 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, CH_2), 2.71 (m, 1H, CH), 2.53 (s, 3H, CH_3), 1.61-1.83 (m, 8H, CH_2). Anal. calcd. for $C_{29}H_{27}ClFN_3O_5$: C, 63.10; H, 4.93; N, 7.61. Found: C, 63.10; H, 4.93; N, 7.61. MS (*m/z*): 553 (M+1).

Table 1. Derivatives of benzisoxazole.

Entry	R (8,9)	Compound 9	Yield, %
1	4-ClC_6H_4	9a	81
2	4-$CMe_3C_6H_4$	9b	76
3	2,4-$Cl_2C_6H_3$	9c	72
4	3-BrC_6H_4	9d	83
5	3,5-$(NO_2)_2C_6H_3$	9e	65
6	3,4,5-$(MeO)_3C_6H_2$	9f	70
7	3-$NO_2C_6H_4$	9g	68
8	4-$MeOC_6H_4$	9h	72
9	2,6-$F_2C_6H_3$	9i	60
10	3-ClC_6H_4	9j	73

2.3. Biological evaluation-antibacterial, antioxidant and anti-inflammatory activities

2.3.1. Antibacterial activity

Antibacterial tests were carried out by disc diffusion method using 100 µL of suspension containing 10^6 cells/mL of bacteria. 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 at 24 h. One antibacterial drug, chloramphenicol was used as positive control. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms and the results are summarised in Table 2.

2.3.2. Antioxidant activity

2.3.2.1. DPPH radical scavenging assay

DPPH radical scavenging assays [15] 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 percentage radical scavenging activity was calculated according to Equation (1).

$$\text{Inhibition (\%)} = (\text{Absorbance control-Absorbance Sample/Absorbance Control}) \times 100 \quad (1)$$

The DPPH radical scavenging activity is demonstrated in Figure 1 and Table 3.

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

Compound	Zone of inhibition in millimetre *				
	Escherichia coli	*Bacillus subtilis*	*Klebsiella pneumoniae*	*Salmonella typhi*	*Shigella flexneri*
9a	12	11	11	10	11
9b	13	12	9	12	9
9c	14	-	10	-	-
9d	10	13	9	12	10
9e	16	15	16	22	26
9f	10	15	11	11	9
9g	11	12	10	9	10
9h	12	-	-	-	-
9i	-	-	10	-	-
9j	17	-	-	11	11

* Inhibition zones including disc (6 mm) diameter, Positive control zone is 35 to 40 mm, "-" = Not active.

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

Compound	IC_{50} values in µM		
	DPPH scavenging assay	Hydroxy radical scavenging assay	Superoxide radical scavenging assay
9a	7.0	7.5	7.3
9b	4.1	3.9	3.8
9c	6.2	6.4	6.5
9d	7.9	8.2	8.2
9e	6.5	6.2	6.2
9f	4.9	4.6	4.1
9g	6.8	6.8	6.8
9h	5.2	5.1	4.7
9i	8.5	8.5	8.5
9j	7.4	8.0	7.8
Ascorbic acid	3.5	3.4	-
Quercetin	-	-	2.8

"-" = Not active.

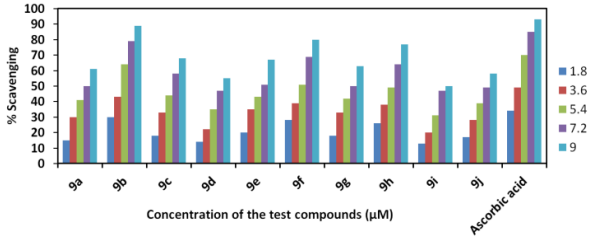

Figure 1. DPPH radical scavenging assay.

2.3.2.2. Hydroxyl radical scavenging assay

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_2O_2 (10 mM), 0.36 mL of deoxyribose (10 mM), 1 mL of the 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) added in sequence. The mixture was incubated at 37 °C for 1 h. 1 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 [16]. Ascorbic acid was used as a positive control. Percentage inhibition was evaluated by using Equation (2).

Inhibition (%) = (Absorbance control-Absorbance Sample/Absorbance Control) × 100 (2)

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

2.3.2.3. Superoxide anion radical scavenging assay

1 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 concentration 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 [17]. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity and it is illustrated in Figure 3 and Table 3.

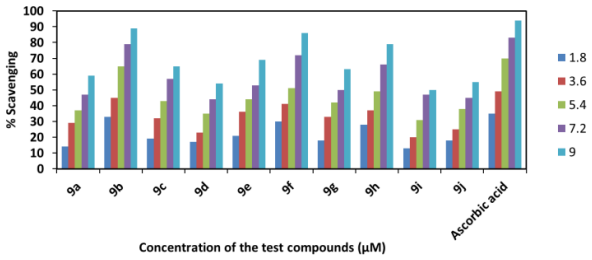

Figure 2. Hydroxyl radical scavenging assay.

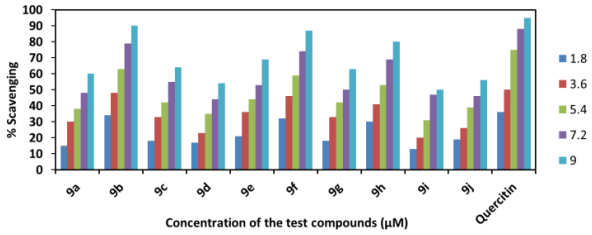

Figure 3. Super oxide radical scavenging assay.

2.3.3. Anti-inflammatory activity

2.3.3.1. Lipoxygenase inhibition assay

Lipoxygenase inhibition assay [18] was carried out using linoleic acid as substrate and 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, solution of compounds **9a-j** in DMSO (1 mg/mL) was added and incubated with the enzyme with various concentrations (1.8-9.0 µM).

Table 4. Anti-inflammatory activity of benzisoxazoles **9a-j**.

Compound	IC_{50} values in µM	
	Lipoxygenase inhibition assay	PLA_2 inhibition assay
9a	8.6	79.0
9b	4.1	37.4
9c	6.2	62.7
9d	7.9	76.9
9e	3.9	40.3
9f	5.0	49.8
9g	3.9	40.5
9h	5.2	53.0
9i	8.5	81.1
9j	7.4	75.3
Indomethacin	3.0	-
Aristolochic acid	-	30.0

"-" = Not determined.

The tubes were agitated and incubated at room temperature for 5 min, after which 2.0 mL of substrate solution, 0.6 mM linoleum 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. Percentage (%) inhibition was calculated by Equation (3).

Inhibition (%) = (Absorbance control-Absorbance Sample/Absorbance Control) × 100 (3)

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

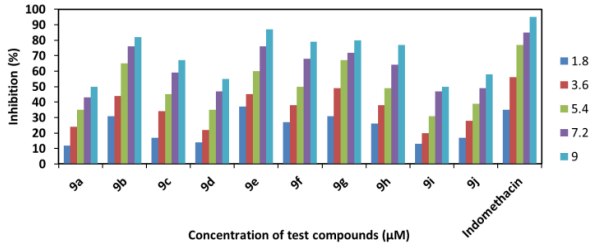

Figure 4. Lipoxygenase inhibition assay.

2.3.3.2. Inhibition of PLA_2 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. 1 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. 10 µg of venom sample (secretory-PLA_2 purchased from sigma) was incubated with various concentration of compounds **9a-j** (1 mg/mL 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 [19] and inhibitory activities are summarised in Figure 5 and Table 4.

3. Results and discussion

Sodium methoxide induced cyclocondensation of *tert*-butyl 4-oxopiperidine-1-carboxylate (**1**) and dimethyl 2-methylene succinate (**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 undergo intramolecular cyclication to form lactone with exocyclic double bond. Later, base induced migration of double bond in to the ring gives compound **3**). Selective hydrolysis of methyl ester group in compound **3** by lithium hydroxide in methonolic water to get 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 to furnish 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 to give 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** to afford final products 8-acyl-4-(4-(6-fluoro benzo[d]isoxazol-3-yl)piperidine-1-carbonyl)-3-methyl-1-oxa-8-azaspiro[4.5]dec-3-en-2-one (**9**) as shown in Scheme 1, Table 1. The structures of the synthesized compounds are established with the help of spectral data.

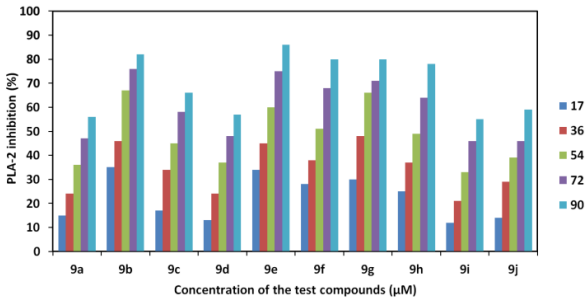

Figure 5. Inhibition of PLA2 induced haemolysis.

Compounds **9a-b** and **9d-g** showed good antibacterial activity against *Escherichia coli*, *Klebsiella pneumonia*, *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. In all the anti-oxidant assays compounds **9b**, **9f** and **9h** containing electron donating groups exhibited good inhibitory activity. The remaining compounds showed moderate anti-oxidant activity. At this stage, it is not possible to give any rational explanation for the anti-oxidant activities of benzisoxazole derivatives even in the absence of essential phenolic group. In both lipoxygenase 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 anti-oxidant activity, whereas those with deactivating groups exhibited anti-inflammatory activities.

4. Conclusion

In summary, we have synthesized a series of new benzisoxazole derivatives in good yields and screened them for antibacterial, antioxidant and anti-inflammatory activity. 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.

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