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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, anti-inflammatory and neuroprotective activity of pyrazole and pyrazolo[3,4-d]pyridazine bearing 3,4,5-trimethoxyphenyl

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Abstract A new series of 3,4,5-trimethoxyphenyl bearing pyrazole **(4a–g)** and pyrazolo[3,4-d]pyridazine **(5a–g)** scaffolds were synthesized in good yield. The newly synthesized compounds were characterized on the basis of elemental and spectroscopic analyses. Their inhibitory activity against the pro-inflammatory inducible nitric oxide synthase and cyclooxygenase-2 proteins expression in lipopolysaccharide-stimulated murine RAW 264.7 macrophages were assessed and showed various potencies. All pyrazolo[3,4-d]pyridazine compounds **(5a–g)** strongly down regulated lipopolysaccharide inducible nitric oxide synthase expression to the range of 20.3 ± 0.6–$51.3 \pm 3.5\%$ relative to the bioactive pyrazole derivatives **4b**, **4c**, **4e** and **4g**. With the exception of inactive compounds **4c** and **4d**, all other synthesized compounds inhibited cyclooxygenase-2 expression below 100% in the lipopolysaccharide-stimulated cells, which being declined maximally to $42.8 \pm 1.4\%$ by one of the pyrazolo[3,4-d]pyridazine compounds **(5d)**. Moreover, the neuroprotective activity of the less cytotoxic compounds **4b**, **(4e–g)** and **(5a–g)** were evaluated against 6-hydroxydopamine (6-OHDA)-induced neuroblastoma SH-SY5Y cell death and exhibited significant ($p < 0.05$) cell protection. The pyrazolo[3,4-d]pyridazine compound **(5e)** exhibited more than 100% of relative neuroprotection ($110.7 \pm 4.3\%$) with an additional advantage of having the highest cell viability index ($107.2 \pm 2.9\%$).

Keywords iNOS · COX-2 · Anti-inflammatory · Neuroprotective · Pyrazole derivatives · Pyrazolo[3,4-d] pyridazine derivatives

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to alleviate inflammation and pain associated with many pathological conditions. Traditional NSAIDs act by inhibiting the cyclooxygenase isoforms (COXs) and hence inhibiting the biosynthesis of prostaglandins from arachidonic acid (Jouzeau et al. 1997; Cairns 2007). COX-2 is inducible in inflammation and carcinogenesis whereas the isoform (COX-1) is constitutively expressed under all physiological conditions. Selective COX-2 inhibitors such as the diaryl-substituted pyrazole derivative have less side effects as compared to traditional NSAIDs (Michaux and Charlier 2004). However, long term use of both traditional NSAIDs and COXIBS has been reported to cause significant cardiovascular side effects (Patel et al. 2004), and thus discovery of new, safe and effective drugs is still a necessity. Pyrazole scaffold possess a broad spectrum of

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applications including anti-inflammatory (Sharma et al. 2010; Tewari et al. 2010; el-Hawash and el-Mallah 1998; Gokhan-Kelekci et al. 2007; Domiati et al. 2016), analgesic (Hall et al. 2008), neuroprotective (Jayaraj et al. 2013), anticonvulsant, and antidepressant activity (Abdel-Aziz et al. 2009; Özdemir et al. 2007). Pyrazolopyridazine derivatives are also reported as good anti-inflammatory agents (Tewari and Mishra 2001; Tewari et al. 2011) and as analgesic in neuropathic pain (Myatt et al. 2010). The literature survey reveals that many pyrazoline derivatives have been used for clinical applications as NSAIDs. The pyrazoline NSAIDs like phenylbutazone, celecoxib, and deracoxib are potent anti-inflammatory and analgesic agents. This motivated us to design and synthesize novel series of 3,4,5-trimethoxyphenyl bearing pyrazole **(4a–g)** and pyrazolo[3,4-d]pyridazine **(5a–g)**, which have been found to possess an interesting profile for anti-COX-2/iNOS expression-related anti-inflammatory and neuroprotective activity.

Materials and methods

Chemistry

Experimental

All the solvents were procured from Merck. The purity of the synthesized compounds was checked by thin layer chromatography (TLC) performed on Silica gel 60 F_{254} coated plates (Merck). UV light was used for the visualization of TLC spots. The Fourier transform infrared spectroscopy (FT-IR) spectra were performed in KBr pellets on a (Spectrum BX) Perkin Elmer FT-IR spectrophotometer. Melting points were checked on a Gallenkamp melting point apparatus, and the thermometer was uncorrected. NMR Spectra were processsed in DMSO-d_6 on a Bruker nuclear magnetic resonance NMR spectrophotometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at the Research Center, College of Pharmacy, King Saud University, Saudi Arabia. Triple quadruple mass spectroscopy was used for the measurement of molecular masses of compounds. The elemental analysis of the compounds was performed on the CHN Elementar (Analysensysteme GmbH, Germany). The elemental analysis for C, H and N were within the limit of ±0.4% of the theoretical values.

*Synthesis of (2E)-3-(dimethylamino)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**2**)*

A mixture of 3,4,5-trimethoxy acetophenone **1** (0.02 mol) and dimethylformamide-dimethylacetal (DMF–DMA) (0.023 mol) was refluxed in solvent free condition for 10 h, then left to cool at room temperature. The precipitate obtained was filtered off, washed with diethyl ether, dried under vacuum and recrystallized from absolute ethanol to give enaminone **2** as orange crystals. Yield: 90%; M.p.: 128–130 °C; IR (KBr): ν_{max}/cm^{-1}: 1638 (C=O), 1543 (C=C), 1118 (C–O); ^{1}H NMR (DMSO–d_6) δ ppm: 7.71 (1H, d, $J = 12$ Hz, -CH), 7.1 (2H, m, Ar–H), 5.8 (1H, d, $J = 12$ Hz, –CH), 3.8 (6H, s, 2 × OCH_3), 3.7 (3H, s, OCH_3), 3.1 (3H, s, N–CH_3), 2.9 (3H, s, N–CH_3); ^{13}C NMR (DMSO–d_6) δ ppm: 184.4 (C=O), 154.1 (Ar–C), 152.4 (Ar–C), 139.8 (Ar–CH), 135.8 (Ar–CH), 104.6 (C=C), 90.7 (C=C), 60.0 (OCH_3), 55.9 (OCH_3), 44.4 (N–CH_3), 37.1 (N–CH_3); MS (ESI) *m/z*: 265.0 $[M]^+$; Analysis: for $C_{14}H_{19}NO_4$, calcd. C 63.38, H 7.22, N 5.28%; found C 63.14, H 7.20, N 5.30%.

*General synthesis of 1-[1-(4-substituted phenyl)-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanones **(4a–g)***

To an equimolar mixture of enaminone **2** (5.0 mmol) and appropriate hydrazonoyl chloride (**3a–g**) (5.0 mmol) in absolute ethanol was added triethylamine (10.0 mmol) and the mixture was refluxed for 4 h. The precipitated solid was filtered off, washed with absolute ethanol, dried under vacuum and recrystallized from ethanol to give corresponding pyrazole derivative **(4a–g)**.

*1-[1-(4-Methoxyphenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanone **(4a)***

Yield: 85%; IR (KBr): ν_{max}/cm^{-1}: 1684 (C=O), 1651 (C=O), 1519 (C=N), 1231 (C-N), 1130 (C–O); ^{1}H NMR (DMSO-d_6) δ ppm: 8.9 (1H, s, pyrazole), 7.9 (2H, d, $J =$ 8.5 Hz, Ar–H), 7.1 (4H, m, Ar–H), 3.6 (12H, s, 4 × OCH_3), 2.59 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 192.7 (C=O), 187.8 (C=O), 158.7 (Ar–C), 152.6 (Ar–C), 149.8 (Ar–C), 142.0 (Ar–C), 132.1 (Ar–C), 122.2 (Ar–C), 121.0 (Ar–C), 114.7 (Ar–C), 106.7 (Ar–C), 60.1 (OCH_3), 56.0 (OCH_3), 27.3 ($COCH_3$); MS (ESI) *m/z*: 411.2 $[M]^+$, 243.0 [100 %]; Analysis: for $C_{22}H_{22}N_2O_6$, calcd. C 64.38, H 5.40, N 6.83% found C 64.13, H 5.41, N 6.84%.

*1-[1-(4-Flourophenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanone **(4b)***

Yield: 90%; IR (KBr): ν_{max}/cm^{-1}: 1684 (C=O), 1635 (C=O), 1506 (C=N), 1233 (C–N), 1132 (C–O); ^{1}H NMR (DMSO-d_6) δ ppm: 9.0 (1H, s, pyrazole), 8.0 (2H, d, $J =$ 8.5 Hz, Ar–H), 7.4 (2H, d, $J = 8.5$ Hz, Ar–H), 7.1 (2H, s, Ar–H), 3.8 (9H, s, 3 × OCH_3), 2.6 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 192.7 (C=O), 187.8 (C=O), 152.7 (Ar–C), 131.8 (Ar–C), 121.7(Ar–C), 116.6 (Ar–C), 106.7 (Ar–C), 60.1 (OCH_3), 56.0 (OCH_3), 27.3 ($COCH_3$);

MS (ESI) *m/z*: 399.2 $[M]^+$; Analysis: for $C_{21}H_{19}N_2O_5F$, calcd. C 63.31, H 4.81, N 7.03% found C 63.55, H 4.82, N 7.01%.

1-[1-(4-Methylyphenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanone ***(4c)***

Yield: 80%; IR (KBr): ν_{max}/cm^{-1}: 1684 (C=O), 1636 (C=O), 1506 (C=N), 1232 (C–N), 1131 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 8.9 (1H, s, pyrazole), 7.9 (2H, d, $J =$ 8.5 Hz, Ar–H), 7.4 (2H, d, $J = 8.5$ Hz, Ar–H), 7.1 (2H, s, Ar–H), 3.7 (9H, s, $3 \times OCH_3$), 3.6 (3H, s, CH_3), 2.6 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 192.7 (C=O), 187.6 (C=O), 156.7 (Ar–C), 152.4 (Ar–C), 149.5 (Ar–C), 141.0 (Ar–C), 132.1 (Ar–C), 122.8 (Ar–C), 121.5 (Ar–C), 114.2 (Ar–C), 106.8 (Ar–C), 60.1 (OCH_3), 56.0 (OCH_3), 27.3 ($COCH_3$) MS (ESI) *m/z*: 395.2 $[M]^+$, 227.0 [100 %]. Analysis: for $C_{22}H_{22}N_2O_5$, calcd. C 66.99, H 5.62, N 7.10%; found C 66.73, H 5.63, N 7.12%.

1-[1-Phenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl] ethanone ***(4d)***

Yield: 70%; IR (KBr): ν_{max}/cm^{-1}: 1685 (C=O), 1664 (C=O), 1506 (C=N), 1233 (C–-N), 1127 (C–O); ^1H NMR (DMSO-d6) δ ppm: 9.0 (1H, s, pyrazole), 8.0 (1H, t, Ar–H), 7.6 (2H, d, $J = 7$ Hz, Ar–H), 7.4 (2H, d, $J = 7$ Hz, Ar–H), 7.1 (2H, s, Ar–H), 3.8 (9H, s, $3 \times OCH_3$), 2.6 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 192.7 (C=O), 187.8 (C=O), 152.7 (Ar–C), 150.1 (Ar–C), 142.0 (Ar–C), 138.6 (Ar–C), 132.4 (Ar–C), 129.7 (Ar–C), 127.9(Ar–C), 122.4(Ar–C), 119.4 (Ar–C), 106.7 (Ar–C), 60.1 (OCH_3), 56.0 (OCH_3), 27.2 ($COCH_3$); MS (ESI) *m/z*: 381.1 $[M]^+$, 213.0 [100 %]; Analysis: for $C_{21}H_{20}N_2O_5$, calcd. C 66.31, H 5.30, N 7.36%; found C 66.56, H 5.32, N 7.34%.

1-[1-(4-Sulfonamidophenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanone ***(4e)***

Yield: 75%; IR (KBr): ν_{max}/cm^{-1}: 3410 (NH_2), 1690 (C=O), 1652 (C=O), 1506 (C=N), 1334 (S=O), 1232 (C–N), 1131 (C-O); ^1H NMR (DMSO-d_6) δ ppm: 9.1 (1H, s, pyrazole), 8.2 (2H, d, $J = 8.5$ Hz, Ar–H), 8.0 (2H, d, $J =$ 8.5 Hz, Ar–H), 7.5 (2H, s, $–NH_2$, D_2O exchang.), 7.1 (2H, m, Ar–H), 3.8 (9H, s, $3 \times OCH_3$), 2.6 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 192.7 (C=O), 187.6 (C=O), 152.7 (Ar–C), 150.6 (Ar–C), 143.0 (Ar–C), 142.1 (Ar–C), 140.6 (Ar–C), 132.3 (Ar–C), 127.3 (Ar–C), 122.8 (Ar–C), 119. (Ar–C)6, 106.8 (Ar–C), 60.1 (OCH_3), 56.0 (OCH_3), 27.3 ($COCH_3$); MS (ESI) *m/z*: 460.2 $[M]^+$; Analysis: for $C_{21}H_{21}N_3O_7S$, calcd. C 54.89, H 4.61, N 9.15S 6.98%; found C 54.67, H 4.62, N 9.14S 6.97%.

1-[1-(4-Bromophenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanone ***(4f)***

Yield: 85%; IR (KBr): ν_{max}/cm^{-1}: 1686 (C=O), 1635 (C=O), 1506 (C=N), 1233 (C–N), 11231 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.1 (1H, s, pyrazole), 8.0 (2H, d, $J =$ 8.5 Hz Ar–H), 7.8 (2H, d, $J = 8.5$ Hz, Ar–H), 7.1 (2H, s, Ar–H), 3.8 (9H, s, $3 \times OCH_3$), 2.6 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 192.7 (C=O), 187.5 (C=O), 153.3 (Ar–C), 132.5 (Ar–C), 121.4 (Ar–C), 106.8 (Ar–C), 60.1 (OCH_3), 56.0 (OCH_3), 27.3 ($COCH_3$); MS (ESI) *m/z*: 460.2 $[M]^+$; Analysis: for $C_{21}H_{19}N_2O_5Br$, calcd. C 54.92, H 4.17, N 6.10%; found C 55.12, H 4.16, N 6.12%.

1-[1-(4-Chlorohenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanone ***(4g)***

Yield: 70%; IR (KBr): ν_{max}/cm^{-1}: 1688 (C=O), 1635 (C=O), 1505 (C=N), 1232 (C–N), 11231 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.0 (1H, s, pyrazole), 8.0 (2H, d, $J =$ 8.5 Hz Ar–H), 7.6 (2H, d, $J = 8.5$ Hz, Ar–H), 7.1 (2H, s, Ar–H), 3.8 (9H, s, $3 \times OCH_3$), 2.6 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 192.7 (C=O), 187.5 (C=O), 153.0 (Ar–C), 129.6 (Ar–C), 121.12 (Ar–C), 106.8 (Ar–C), 60.0 (OCH_3), 56.0 (OCH_3), 25.0 ($COCH_3$); MS (ESI) *m/z*: 416.0 $[M]^+$; Analysis: for $C_{21}H_{19}N_2O_5Cl$, calcd. C 60.80, H 4.62, N 6.75 %; found C 60.65, H 4.61, N 6.73 %.

General synthesis of pyrazolo[3,4-d]pyridazine derivatives ***(5a–g)***

A mixture of pyrazole derivative **(4a–g)** (3.0 mmol) and hydrazine hydrate 99% (3.0 mmol) in absolute ethanol (30 mL) was refluxed for 1 h and then cooled at room temperature. The product that was separated out was filtered, dried under vacuum and recrystallized from ethanol.

7-Methyl-2-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine ***(5a)***

Yield: 80%; IR (KBr): ν_{max}/cm^{-1}: 1507 (C=N), 1264 (C–N), 1126 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.5 (1H, s, pyrazole), 8.1 (2H, d, $J = 9$ Hz, Ar–H), 7.3 (2H, s, Ar–H), 7.2 (2H, d, $J = 9$ Hz, Ar–H), 3.8 (9H, s, $3 \times OCH_3$), 3.6 (3H, s, OCH_3), 2.8 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 159.7 (Ar–C), 153.4 (Ar–C), 150.8 (Ar–C), 143.6 (Ar–C), 139.0 (Ar–C), 132.5 (Ar–C), 132.0 (Ar–C), 124.5 (Ar–C), 122.9 (Ar–C), 115.2 (Ar–C), 114.7 (Ar–C), 105.5 (Ar–C), 60.0 (OCH_3), 56.0 (OCH_3), 55.6 (OCH_3), 18.0 (CH_3); MS (ESI) *m/z*: 407.2 $[M]^+$; Analysis: for $C_{22}H_{22}N_4O_4$, calcd. C 65.01, H 5.46, N 13.78%; found C 65.26, H 5.45, N 13.74%.

7-Methyl-2-(4-flourophenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine ***(5b)***

Yield: 90%; IR (KBr): ν_{max}/cm^{-1}: 1507 (C=N), 1220 (C–N), 1127 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.6 (1H, s, pyrazole), 8.2 (2H, d, $J = 9$ Hz, Ar–H), 7.5 (2H, d, $J = 9$ Hz, Ar–H), 7.3 (2H, s, Ar–H), 3.7 (9H, s, $3 \times OCH_3$), 2.8 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 153.2 (Ar–C), 139.1 (Ar–C), 131.9 (Ar–C), 125.4 (Ar–C), 123.8 (Ar–C), 116.7 (Ar–C), 115.2 (Ar–C), 105.5 (Ar–C), 60.0 (OCH_3), 56.0 (OCH_3), 18.0 (CH_3); MS (ESI) *m/z*: 395.2 $[M]^+$, 102.3 [100%]; Analysis: for $C_{21}H_{19}N_4O_3F$, calcd. C 63.95, H 4.86, N 14.21%; found C 63.75, H 4.87, N 13.25%.

7-Methyl-2-(4-methylphenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine ***(5c)***

Yield: 85%; IR (KBr): ν_{max}/cm^{-1}: 1507 (C=N), 1229 (C–N), 1127 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.0 (1H, s, pyrazole), 8.1 (2H, d, $J = 9$ Hz, Ar–H), 7.5 (2H, d, $J = 9$ Hz, Ar–H), 7.3 (2H, s, Ar–H), 3.6 (9H, s, $3 \times OCH_3$), 2.6 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 153.1 (Ar–C), 139.0 (Ar–C), 131.7 (Ar–C), 125.2 (Ar–C), 123.5 (Ar–C), 116.5 (Ar–C), 115.0 (Ar–C), 105.1 (Ar–C), 60.1 (OCH_3), 56.0 (OCH_3), 18.0 (CH_3); MS (ESI) *m/z*: 391.3 $[M]^+$, 254.9 [100%]; Analysis: for $C_{22}H_{22}N_4O_3$, calcd. C 67.68, H 5.68, N 14.35%; found C 67.87, H 5.67, N 14.30%.

7-Methyl-2-phenyl-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine ***(5d)***

Yield: 80%; IR (KBr): ν_{max}/cm^{-1}: 1506 (C=N), 1231 (C–N), 1128 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.6 (1H, s, pyrazole), 8.2 (2H, d, $J = 7$ Hz, Ar–H), 7.7 (1H, t, $J = 7.5$ Hz, Ar–H), 7.6 (2H, d, $J = 7$ Hz, Ar–H), 7.4 (2H, s, Ar–H) 4.0 (6H, s, $2 \times OCH_3$), 3.8 (3H, s, $3 \times OCH_3$), 2.9 (3H, s, $COCH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 153.6 (Ar–C), 153.2 (Ar–C), 151.0 (Ar–C), 143.8 (Ar–C), 139.2 (Ar–C), 131.9 (Ar–C), 129.7 (Ar–C), 125.4 (Ar–C), 121.5 (Ar–C), 115.2 (Ar–C), 105.5 (Ar–C), 60.0 (OCH_3), 56.0 (OCH_3), 18.0 (CH_3); MS (ESI) *m/z*: 377.2 $[M]^+$; Analysis: for $C_{21}H_{20}N_4O_3$, calcd. C 67.01, H 5.36, N 14.88%; found C 67.27, H 5.37, N 14.92%.

7-Methyl-2-(4-sulfonamidophenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine ***(5e)***

Yield: 75%; IR (KBr): ν_{max}/cm^{-1}: 3354 (NH_2), 1506 (C=N), 1334 (S=O), 1239 (C–N), 1134 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.7 (1H, s, pyrazole), 8.4 (2H, d, $J = 8.5$ Hz, Ar–H), 8.0 (2H, d, $J = 8.5$ Hz, Ar–H), 7.5 (2H, s, $-NH_2$, D_2O exchang.), 7.3 (2H, m, Ar–H), 3.9 (6H, s, $2 \times OCH_3$), 3.7 (3H, s, OCH_3), 2.9 (3H, s, $-CH_3$); ^{13}C NMR (DMSO-d_6) δ ppm: 153.7 (Ar–C), 153.2 (Ar–C), 151.2 (Ar–C), 144.3 (Ar–C), 141.2 (Ar–C), 139.2 (Ar–C), 131.8 (Ar–C), 127.2 (Ar–C), 126.0 (Ar–C), 121.9 (Ar–C), 115.4 (Ar–C), 105.6 (Ar–C), 60.1 (OCH_3), 56.1 (OCH_3), 18.0 (CH_3); MS (ESI) *m/z*: 456.2 $[M]^+$; Analysis: for $C_{21}H_{21}N_5O_5S$, calcd. C 55.37, H 4.65, N 15.38S 7.04%; found C 55.58, H 4.66, N 15.42S 7.05%.

7-Methyl-2-(4-bromophenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine ***(5f)***

Yield: 82%; IR (KBr): ν_{max}/cm^{-1}: 1497 (C=N), 1233 (C–N), 1128 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.7 (1H, s, pyrazole), 8.2 (2H, d, $J = 8.5$ Hz, Ar–H), 7.9 (2H, d, $J = 8.5$ Hz, Ar–H), 7.4 (2H, s, Ar–H), 4.0 (9H, s, $3 \times OCH_3$), 2.9 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 153.1 (Ar–C), 153.0 (Ar–C), 151.5 (Ar–C), 144.6 (Ar–C), 141.0 (Ar–C), 139.1 (Ar–C), 131.6 (Ar–C), 127.2 (Ar–C), 126.2 (Ar–C), 121.0 (Ar–C), 115.3 (Ar–C), 105.1 (Ar–C), 60.0 (OCH_3), 56.0 (OCH_3), 18.0 (CH_3); MS (ESI) *m/z*: 456.2 $[M]^+$; Analysis: for $C_{21}H_{19}N_4O_3Br$, calcd. C 55.40, H 4.21, N 12.31%; found C 55.62, H 4.20, N 12.35%.

7-Methyl-2-(4-chlorophenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine ***(5g)***

Yield: 85%; IR (KBr): ν_{max}/cm^{-1}: 1505 (C=N), 1233 (C–N), 1127 (C–O); ^1H NMR (DMSO-d_6) δ ppm: 9.7 (1H, s, pyrazole), 8.3 (2H, d, $J = 9$ Hz, Ar–H), 7.8 (2H, d, $J = 9$ Hz, Ar–H), 7.4 (2H, s, Ar–H), 4.0 (9H, s, $3 \times OCH_3$), 2.9 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ ppm: 153.2 (Ar–C), 138.0 (Ar–C), 129.7 (Ar–C), 123.2 (Ar–C), 105.6 (Ar–C), 60.0 (OCH_3), 56.1 (OCH_3), 18.0 (CH_3); MS (ESI) *m/z*: 412.0 $[M]^+$; Analysis: for $C_{21}H_{19}N_4O_3Cl$, calcd. C 61.39, H 4.66, N 13.64%; found C 61.50, H 4.65, N 13.60%.

Biological assays

Cell cultures

The murine RAW 264.7 macrophage and SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, MD, USA). The macrophage cells were cultured in Dulbecco's modified essential medium (DMEM, Invitrogen-Gibco, CA, USA) containing 10% inactivated fetal bovine serum (FBS, Sigma-Aldrich, MO, USA). The neuroblastoma cells were grown in a 1:1 DMEM and Hams F12 nutrient medium (Invitrogen-Gibco) containing 10% heat-inactivated FBS. Each cell line was incubated at 37 °C in a humidified 5% CO_2, 95% air incubator.

Cell viability assay

Human neuroblastoma SH-SY5Y or murine RAW 264.7 macrophage cells were seeded in 96-well culture plates at an initial density of 3×10^4 cells/well (Corning, NY, USA). The testing compound at 1 and 10 µM was incorporated into the cell culture in DMEM medium in humidified incubator within a 5% CO_2 atmosphere at 37 °C for 24 h. The survival of the cells was determined using Alamar blue dye (Invitrogen, CA, USA) according to the manufacturer instruction. The absorbance (A) was measured at 570 nm using a microplate reader. This cell viability assay is similar in principle to that of 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MMT) which has been previously validated by Bohlken et al. 2009.

In vitro anti-inflammatory assay

By using murine RAW 264.7 macrophages and a bacterial lipopolysaccharide (LPS), the in vitro anti-inflammatory activity of compounds (10 µM) was performed according to the previously described assay (Ahmed et al. 2006) modified from the method of Park et al. 2005. The expression of iNOS, COX-2 proinflammatory proteins in the LPS-stimulated cells was determined by western-immunoblot analysis after being separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. β-actin was used as an internal loading control to normalize the blot data.

In vitro neuroprotective assay

Human neuroblastoma SH-SY5Y cells were seeded in 96-well culture (*vide supra*), treated with the tested compounds at 1 µM for 1 h and then exposed to the neurotoxin 6-hydroxydopamine (6-OHDA, Sigma-Aldrich, MO, USA) at 20 µM. Following a further 18 h incubation period, SH-SY5Y cells survival was determined using Alamar blue assay by measuring the produced pink dye at 595 nm. Relative protection (%) was calculated as [(optical density (OD) of 6-OHDA/compounds-treated cells −OD of 6-OHDA-treated cells) / (OD of control cells − OD of 6-OHDA-treated cells)].

Results

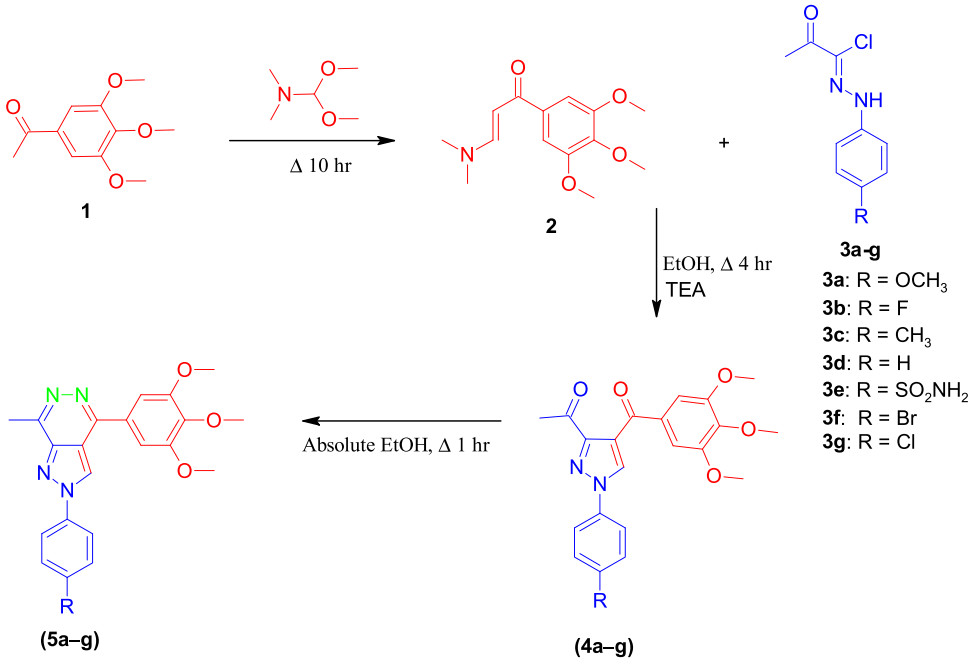
Chemistry

Treatment of 3,4,5-trimethoxy acetophenone **1** with (DMF-DMA) under solvent free condition, afforded a orange crystalline product identified as (2*E*)-3-(dimethylamino)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one **2**. The structure of the product was identified by elemental analysis and spectral data. ^{1}H NMR spectrum displayed two singles at δ_H 2.93, 3.14 ppm due to the *N,N*-dimethyl protons and two doublets at δ_H 5.83 and 7.71 ppm (d, $J = 12$ Hz) due to the ethylenic protons, in addition to the multiplet at the region δ_H 7.18 ppm (2H, m, aromatic). The value of coupling constant ($J = 12$ Hz) for the ethylenic protons indicate that the enaminone **2** exist in the *E*-configuration, which is in complete agreement with the results recently reported (Shaaban et al. 2007). The reaction of enaminone **2** with the nitrilamine, generated in situ by the reaction of triethylamine on the (1*E*)-*N*-(4-methoxyphenyl)-2-oxopropanehydrazonoyl chloride **3a** in refluxing absolute ethanol afforded a single product (1-[1-(4-methoxyphenyl-4-(3,4,5-trimethoxybenzoyl)-1H-pyrazol-3-yl]ethanone **4a**. ^{1}H NMR spectrum of the isolated product **4a** revealed three singlets at δ_H 2.59, 3.34 and 8.94 ppm due to acetyl-CH_3, methoxy-CH_3 and pyrazole-5-CH protons, respectively, in addition to aromatic protons (6H, m) in the region of δ 7.12–7.93 ppm. The structure of **4a** was further confirmed by its reaction with hydrazine hydrate 99% to afford a white crystalline product, which was identified as 7-methyl-2-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-2*H*-pyrazolo[3,4-d]pyridazine (**5a**) (Scheme 1). IR spectrum of **4a** showed two carbonyl absorption bands at λ_{max} 1684 and 1651 cm^{-1} which disappeared in IR spectrum of **5a**. The ^{1}H NMR spectrum of the isolated product revealed a singlet at δ_H 9.52 ppm, which indicates the presence of pyrazole 5-CH proton (Hamdy and El-Senousy 2013). Also the ^{13}C NMR of compound **5a** is in agreement with its chemical structure. Under the same experimental conditions, the enaminone **2** was allowed to react with hydrazonoyl chlorides **(3b–g)** to afford the corresponding pyrazole derivatives **(4b–g)**. The latter product underwent cyclocondensation upon treatment with hydrazine hydrate 99% in refluxing ethanol to yield pyrazolo[3,4-d]pyridazine derivatives **(5b–g)**. All the structures were confirmed on the basis of spectral data and elemental analysis. The data of the physiochemical properties of all compounds is given in (Table 1).

The anti-inflammatory activity

Gram-negative bacterial LPS is a key component linked to septic shock which is associated by a buildup of inflammatory mediators such as prostaglandins and NO (Hewett and Roth 1993) due to overexpression of the proinflammatory COX-2 and iNOS proteins, respectively. Therefore, management of the inflammatory responses is achievable by compounds which can down regulate the LPS-induced expression of COX-2 or iNOS or both proteins in the affected tissues. In this work, LPS-stimulated RAW264.7 macrophages and immunoblot analysis were employed to recognize which compounds possess an

Scheme 1 Synthetic route of compounds **(4a–g)** and **(5a–g)**



in vitro inhibitory effect on the expression of proinflammatory proteins. The well-known anti-inflammatory drug dexamethasone was used as a positive control since it inhibits iNOS and COX-2 expression (Korhonen et al. 2002; Koistinaho et al. 1999; Oh et al. 2015). Before the anti-inflammatory assay, compounds **(4a–g)** and **(5a–g)** were proved to be non-cytotoxic based on cell viability (>90%) when assessed against the growth of RAW264.7 macrophage cells via alamar blue assay at the same concentration (10 µM).

The effect of pyrazoline and pyrazolo[3,4-d]pyridazine derivatives on LPS-induced iNOS expression

In this experiment, the synthetic compounds exerted significant ($p < 0.05$) down regulation on the LPS-induced iNOS expression, except compounds **4a**, **4d** and **4f** which showed no activity. Based on whether the compounds belong to pyrazole or pyrazolo[3,4-d]pyridazine derivatives, two levels of iNOS expression inhibitory activity have been observed. All pyrazolo[3,4-d]pyridazine derivatives **(5a–g)** strongly inhibited the iNOS expression in the LPS-stimulated cells to less than 51.3% while the active pyrazole derivatives showed moderate anti-iNOS-based anti-inflammatory effect in the range of 58.1 ± 3.7–73.9 ± 6.3% at the same concentration (10 µM) (Fig. 1). In contrast to the inactive compounds **4a**, **4d**, and **4f** regarding the effect on the LPS-induced iNOS expression, their correspondent pyrazolo[3,4-d]pyridazine derivatives **5a**, **5d**, and **5f** disclosed strong inhibition (41.8 ± 1.4, 28.2 ± 8.0, and 20.3 ± 0.6%, respectively). The pyrazolo[3,4-d]pyridazine derivatives having 4-fluorophenyl and 4-chlorophenyl moieties **5b** and **5g** reduced the anti-iNOS expression by 2.5-fold and by more than 1/3 potency of dexamethasone in comparison to their corresponding pyrazole derivatives **4b** and **4g** (Fig. 1). The reaction of hydrazine hydrate with the pyrazole derivatives **4c** and **4e** could escalate the iNOS inhibitory activity by 14.0 and 12.6% as demonstrated by the effect of compounds **5c** and **5e**, respectively.

Table 1 Physico-chemical properties of compounds **(4a–g)** and **(5a–g)**

Compound	R	MP (°C)	M. formula	M. weight
4a	OCH_3	205–207	$C_{22}H_{22}N_2O_6$	410.4
4b	F	235–237	$C_{21}H_{19}FN_2O_5$	398.3
4c	CH_3	225–227	$C_{22}H_{22}N_2O_5$	394.4
4d	H	198–200	$C_{21}H_{20}N_2O_5$	380.3
4e	SO_2NH_2	237–239	$C_{21}H_{21}N_3O_7S$	459.4
4f	Br	240–242	$C_{21}H_{19}BrN_2O_5$	459.2
4g	Cl	245–247	$C_{21}H_{19}ClN_2O_5$	414.8
5a	OCH_3	230–232	$C_{22}H_{22}N_4O_4$	406.4
5b	F	255–257	$C_{21}H_{19}FN_4O_3$	394.3
5c	CH_3	250–252	$C_{22}H_{22}N_4O_3$	390.4
5d	H	203–205	$C_{21}H_{20}N_4O_3$	376.4
5e	SO_2NH_2	315–317	$C_{21}H_{21}N_5O_5S$	455.4
5f	Br	260–262	$C_{21}H_{19}BrN_4O_3$	455.3
5g	Cl	265–267	$C_{21}H_{19}ClN_4O_3$	410.8

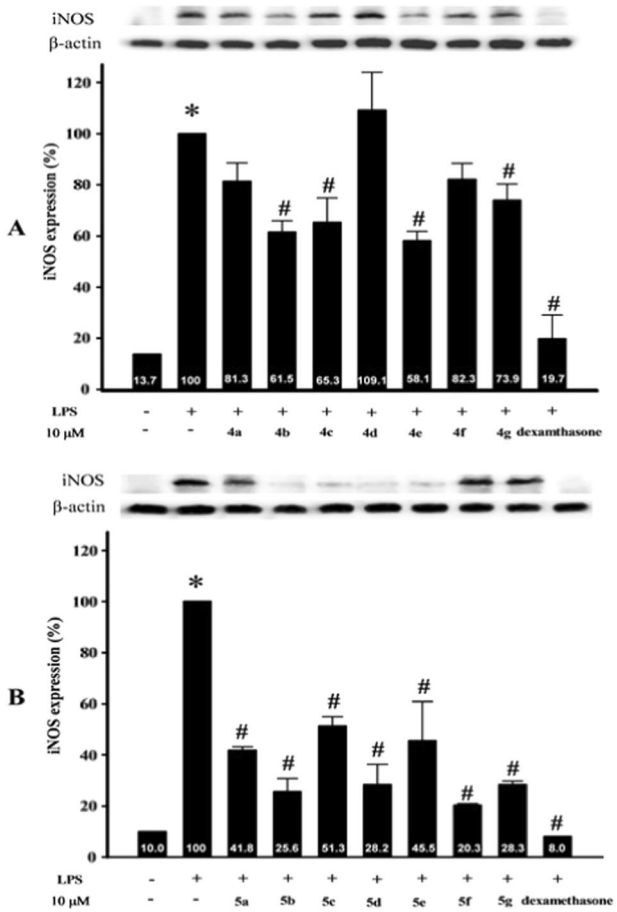


Fig. 1 Effect of 3,4,5-trimethoxybenzene bearing pyrazole **a** and pyrazolo [3,4-d]pyridazine **b** scaffolds on iNOS protein expression in LPS-stimulated RAW264.7 macrophage cells by immunoblot analysis. The values are mean ± S.E.M. ($n = 6$). Equal loading of proteins was verified by β-actin immunonblot. Relative intensity of the LPS-stimulated control group was taken as 100%. Significantly different from untreated control group ($*p < 0.05$) and from LPS-stimulated group ($\#p < 0.05$)

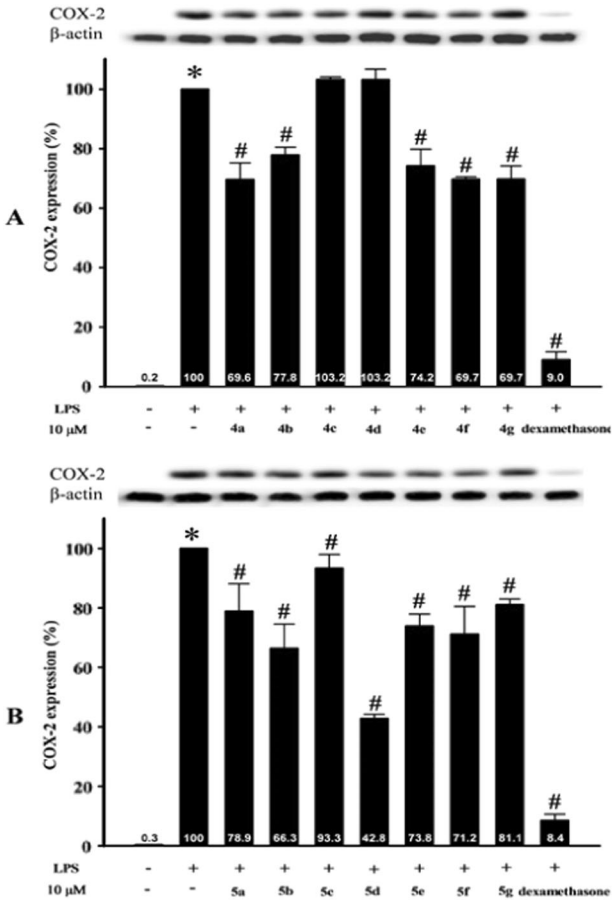


Fig. 2 Effect of 3,4,5-trimethoxybenzene bearing pyrazole **a** and pyrazolo[3,4-d]pyridazine **b** scaffolds on COX-2 protein expression in LPS-stimulated RAW264.7 macrophage cells by immunoblot analysis. The values are mean ± S.E.M. ($n = 6$). Equal loading of protiens was verified by β-actin immunoblot. Relative intensity of the LPS-stimulated control group was taken as 100%. Significantly different from the untreated control group ($*p < 0.05$) and from LPS-stimulated group ($\#p < 0.05$)

The effect of pyrazoline and pyrazolo[3,4-d]pyridazine derivatives on LPS-induced COX-2 expression

The 3,4,5-trimethoxybenzene bearing pyrazole (**4a–g**) and pyrazolo[3,4-d]pyridazine (**5a–g**) derivatives had been assessed for their inhibitory effect on COX-2 expression in LPS-stimulated cells. Among the fourteen tested compounds, only two pyrazole derivatives **4c** and **4d** were devoid of anti-COX-2 expression activity. However, the corresponding pyrazolo[3,4-d]pyridazine derivatives **5c** and **5d**, produced upon condensation with hydrazinehydrate with pyrazole derivatives (Scheme 1) weakly and strongly down regulate the COX-2 expression to the level of 93.3 ± 4.7% and 42.8 ± 1.4% ($p < 0.05$), respectively, at 10 μM relative to that expressed (100%) in the LPS-stimulated control cells. Other pyrazole derivatives having 4-methoxyphenyl **4a**, 4-halophenyl **4b**, **4f**, and **4g**, and 4-sulfonamidophenyl **4e** moieties together with their corresponding pyrazolo[3,4-d]pyridazine compounds **5a**, **5b**, **5f**, **5g**, and **5e** exerted moderate activity by inhibiting the LPS-induced COX-2 expression to the range of 66.3 ± 8.2 to 81.1 ± 1.9% ($p < 0.05$) (Fig. 2).

The neuroprotective activity

A neuroprotective assay based on the protection potential of the tested compound against 6-OHDA (20 μM)-induced toxicity in a human dopaminergic neuroblastoma SHSY5Y cells was performed as reported previously (Lee et al. 2006; Chen et al. 2012). Prior to carrying out this assay, the cytotoxic activity of the synthetic pyrazole, pyrazolo[3,4-d] pyridazine derivatives, and 6-OHDA was measured at the same concentration (1.0 μM) against SH-SY5Y cells, via alamar blue assay. The compounds which showed cell

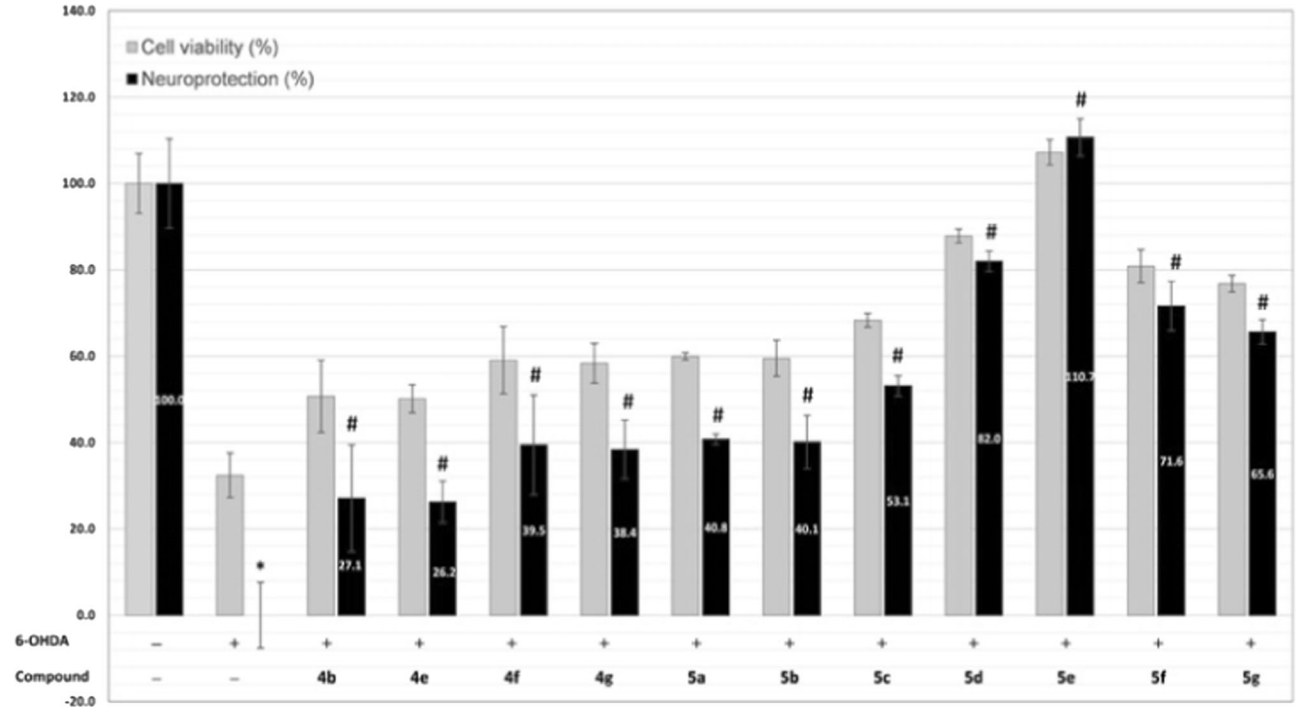


Fig. 3 Effect of selected 3,4,5-trimethoxybenzene bearing pyrazole (**4b** and **4e–4g**) and pyrazolo[3,4-d] pyriadazine (**5a–5g**) scaffolds on neuroblastoma SH-SY5Y cells at 1.0 μM and on 6-OHDA (20 μM) treated neuroblastoma SH-SY5Y cells. Cell viabiliaty was determined for 6-OHDA and tested compounds at 1.0 μM. The values are mean ± S.E.M. ($n = 6$). Significantly different from untreated control group (*$p < 0.05$) and from 6-OHDA (20 μM)-treated control (#$p < 0.05$)

viability >50% at 1.0 μM were selected and then evaluated for their protection potential against 6-OHDA-induced toxicity in SH-SY5Y cells (Fig. 3). Compounds **4a**, **4c**, and **4d** were thus excluded from the neuroprotective assay as they resulted in very low cell viability <40% at 1.0 μM.

The neuroprotective activity of pyrazole and pyrazolo [3,4-d]pyridazine derivatives against 6-OHDA-induced SH-SY5Y cell death

From the results shown in Fig. 3, the pryrazole **4b**, **(4e–g)** and pyrazolo[3,4-d]pyridazine **(5a–g)** derivatives demonstrated significant ($p < 0.05$) neuroprotective activity against 6-OHDA (20 μM)-induced SH-SY5Y cell death with variable potencies (>25% protection) relative to the 6-OHDA (20 μM)-intoxicated control (0.0% protection). Compound **5e**: 7-methyl-2-(4-sulfonamidophenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine exhibited more than 100% relative neuroprotection (110.7 ± 4.3%) against 6-OHDA toxicity in human neuroblastoma cells, followed by the related pyrazolo[3,4-d]pyridazine derivatives **5d**, **5f**, and **5g** in decreasing order (82.0 ± 2.4, 71.6 ± 5.7, 65.6 ± 2.8%, respectively) in the same experimental condition at 1.0 mM. It was also observed that the cytotoxicity of 6-OHDA on SH-SY5Y cells could be reduced in a moderate degree (27.1 ± 12.4 to 53.1 ± 2.4%) by pretreatment **4b**, **(4e–g)**, and **(5a–c)** at 1.0 μM (Fig. 3). However, the higher cell viability index achieved by **5e**, **5d**, **5f**, and **5g** (107.2 ± 2.9, 87.8 ± 1.6, 80.8 ± 3.8, 76.8 ± 1.9%, respectively) relative to **4b**, **(4e–g)** and **(5a–c)** represent an advantageous merit added to their neuroprotective effect.

Discussion

Recent research shows that chronic inflammatory diseases, share common pathways of cellular and molecular dysregulation. Neuroinflammation is also known to have a major role in neuronal damage in neurological and neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (Skaper 2007). Altered gene expression induced by epigenetic mechanisms contributes further to the development of chronic inflammation (Garn et al. 2016). These facts encourage scientists to develop new preventive and therapeutic strategies to effectively control inflammation. In this work, we have constructed novel series of pyrazole **(4a–g)** and pyrazolo [3,4-d]pyridazine **(5a–g)** scaffolds bearing trimethoxyphenyl through condensation of a trimethoxyarylenaminone **2** with seven *N*-substituted arylhydrazonoyl

chlorides **(3a–g)** in presence of triethyl amine (TEA) and then hydrazine, respectively (Scheme 1). Compounds **(4a–g)** and **(5a–g)** thus possessed 1,3,4-trisubstituted pyrazole nucleus. Since celecoxib (1,3,5-trisubstituted pyrazole), its analogous and related compounds (including 1,3,4-trisubstituted pyrazoles) showed anti-inflammatory effects by different mechanisms and indices (Alegaon et al. 2014; Ragab et al. 2013; Villa et al. 2016), the anti-inflammatory activity of compounds **(4a–g)** and **(5a–g)** were tested in vitro. The inhibitory potential of the compounds against the expression of iNOS and COX-2 protein in the LPS-stimulated RAW264.7 macrophages and against 6-OHDA-induced cytotoxicity in neuroblastoma SH-SY5Y cells were measured and considered as indices for the anti-inflammatory activity. The tested pyrazolo[3,4-d]pyridazine compounds **(5a–g)** exerted much stronger down regulation effect against the proinflammatory iNOS protein expression in the LPS-stimulated macrophages to the range of 20.3–51.3% relative to the correspondent pyrazole derivatives **(4a–g)**, which showed moderate activity (58.1–73.9%) while compounds **4a**, **4d**, and **4f** were found to be inactive (Fig. 1). Moreover, except of the inactive compounds **4c** and **4d**, the tested compound showed anti-COX-2-based activity (Fig. 2) but with variable potencies. Among them, the pyrazolo[3,4-d]pyridazine derivative **5d** exhibited the strongest potential to suppress the COX-2 expression to the level of 42.8% relative to that expressed (100%) in the LPS-stimulated control cells. The anti-COX-2 and/or iNOS-based anti-inflammatory activity shown by the synthetic compounds has encouraged us to measure their neuroprotective activity against 6-OHDA-induced toxicity in a human dopaminergic neuroblastoma SHSY5Y cells. Only compounds which showed good cell viability **4b**, **(4e–g)** and **(5a–g)** were assessed and fortunately showed positive activity with variable potencies. The pyrazolo[3,4-d]pyridazine derivative 7-Methyl-2-(4-sulfonamidophenyl)-4-(3,4,5-trimethoxyphenyl)-2H-pyrazolo[3,4-d]pyridazine **5e** displayed >110% neuroprotection against 6-OHDA-inducedtoxicity in SHSY5Y cells relative to the 6-OHDA-intoxicated control. On the basis of the above findings, newly synthesized 1,3,4-substituted pyrazoles **(4a–g)** and its corresponding pyrazolo[3,4-d]pyridazines **(5a–g)** could be considered as suitable templates in a drug discovery program of novel NSAIDs in resolution of chronic endotoxin-induced inflammation and in combatting chemically induced neurotoxicity. However, the effect of the compounds on the cellular mechanisms of COX-2 and iNOS expression, e.g., by inhibiting the activity of the two enzymes or suppression of the activation of the nuclear factor (NF)-κB is worth to be examined. Moreover, the results of neuroprotectivity of compound **5e** deserve to be extended in vivo for evaluation of its therapeutic potential against neurodegenerative diseases, e.g., AD and PD.

Conclusion

In conclusion, novel series of pyrazole and pyrazolo[3,4-d] pyridazine derivatives of 3,4,5-trimethoxyphenyl were synthesized. The reactions were clean and products were obtained as single spot. All the compounds gave satisfactory spectral data. The compounds were evaluated for anti-inflammatory and neuroprotective activities. The pyrazolo [3,4-d]pyridazine derivative **5d** strongly down regulated the LPS-induced COX-2 expression. Furthermore, the pyrazolo [3,4-d]pyridazine derivatives **5a**, **5d**, and **5f** disclosed strong inhibition in the LPS-induced iNOS expression. Compound **5e** exhibited potent neuroprotection against 6-OHDA toxicity in human neuroblastoma cells. From the results of neuroprotectivity, we suggest that further investigation of **(5a–g)** for their therapeutic potential against neurodegenerative diseases is worthy.

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Conflict of interest The authors declare that they have no competing interests.

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