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Design and development of an isatin-1,2,3-triazole hybrid analogue as a potent anti-inflammatory agent with enhanced efficacy and gene expression modulation†

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Isatin (1*H*-indole-2,3-dione) and its derivatives have been found to exhibit various biological activities, including anticancer and antidiabetic properties. In this study, a series of nine isatin-1,2,3-triazole conjugates were synthesized and evaluated for their anti-inflammatory potential *via in vitro* experiments. Their synthesis involved the propargylation of isatin **1** with propargyl bromide to obtain *N*-propargyl isatin **2**, which was subjected to click reactions with different aromatic azides to yield isatin-*N*-1,2,3-triazoles (**3a–i**). The structures of all the compounds were confirmed *via* NMR and HR-MS. The final isatin analogues were tested for their ability to attenuate the production of proinflammatory cytokines in the lipopolysaccharide (LPS)-induced human leukemia monocytic THP-1 cells. Importantly, none of the compounds had any negative effect on THP-1 cell viability at the tested concentrations of 4 mM and 8 mM. LPS induced the production of the cytokines: Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) by 351.4, 7.9 and 14.3 fold, respectively, in THP-1 cells. However, treatment with compound **3e** markedly attenuated the levels of TNF-α (by 6.6 fold and 1.5 fold), IL-6 (by 1.03 fold and 1.41 fold) and MCP-1 (by 3.3 fold and 1.7 fold) by several fold at concentrations of 4 mM and 8 mM, respectively. Furthermore, in the gene expression modulation studies, **3e** was found to downregulate the genes responsible for the production of TNF-α (24 and 25 fold), IL-6 (148 and 502 fold) and MCP-1 (50 and 25 fold) at the two tested concentrations compared with their expression in the LPS-induced THP-1 cells (135 fold, 6612 fold, and 68.8 fold, respectively). Thus, **3e** markedly attenuated the secretion of TNF-α, IL-6 and MCP-1 from LPS-treated THP-1 cells, and also the expression of the concerned genes. At the lowest dose tested, *i.e.*, 4 mM, **3e** had the greatest effect on both gene expression and marker secretion.

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1 Introduction

Isatin (1*H*-indole-2,3-dione) and substituted isatins are heterocyclic molecules found in plants such as *Isatis* spp,[1] *Calanthe discolor*,[2] *Couroupita guianensis*,[3] and *Melochia tomentosa*,[4] bacteria such as *Streptomyces albus*[5] and fungi such as *Chaetomium globosum*.[6] Isatin is a versatile substrate that can be used to prepare a variety of heterocyclic compounds, such as indoles and quinolones.[7] Furthermore, Schiff bases and Mannich bases of isatin are known to possess a wide range of pharmacological properties including antibacterial **I**,[8] anticonvulsant,[9] anti-HIV **II**,[10] antifungal **III**,[11] antiviral **IV**[12] and anti-TB[13] activities. The bis-imine of isatin has shown antimicrobial properties[14] and affected cell viability[15] (Fig. 1). Isatin is used as an intermediate in the synthesis of drugs such as diclofenac and pirquinozol.

The construction of molecular hybrids of potential bioactives using differently substituted nitrogen-rich heterocyclic pharmacophores such as 1,2,3-triazoles has enabled the production of new and biologically promising drugs.[16–18] Chemically, 1,2,3-triazole is stable against acidic/basic hydrolysis and oxidative/reductive conditions, thus reflecting high aromatic stabilization and relative resistance to metabolic degradation.[19–21] 1,2,3-Triazole is one of the key structural units found in a large variety of bioactive molecules such as antifungal,[22] antibacterial,[23,24] antiallergic,[25] anti-HIV,[26,27] antitubercular[28,29] and anti-inflammatory[30] agents. For example, triazole nucleoside derivative **V**[31] inhibited *Mycobacterium*

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† Electronic supplementary information (ESI) available: The supplementary material includes experimental data and spectroscopy data of all the synthesized compounds such as IR, NMR and mass spectra. See DOI: https://doi.org/10.1039/d4ra07294d

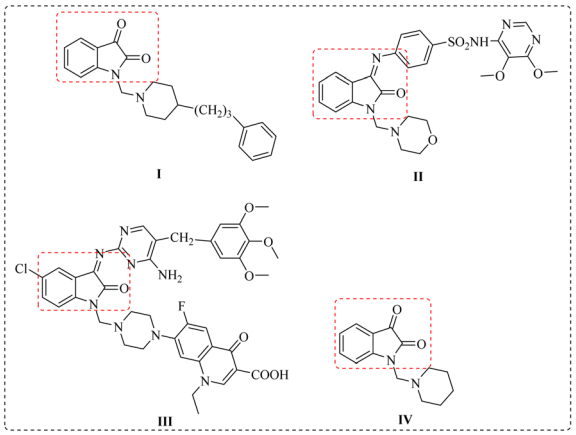


Fig. 1 Structures of some reported bioactive isatin derivatives.

tuberculosis, which disrupts siderophore biosynthesis. Carboxyamidotriazole **VI** is an angiogenesis inhibitor useful in cancer therapy,[32] and 1,2,3-triazole-containing compound **VII** is a potential dual-action HIV-1 protease and nonnucleoside reverse transcriptase inhibitor.[33] The cephalosporin analogue cefatrizine **VIII** is also an antibiotic used to treat bacterial infections of the urinary tract, liver and gallbladder, *etc.*[34,35] (Fig. 2).

Our earlier research on the synthesis of novel hybrid analogues such as piperonal-thiazolidinedione-triazoles,[36] chromanochalcone-triazoles,[37] andrographolide-triazoles[38] resulted in the production of potential anticancer, hypolipidemic and antidiabetic agents. In continuation, herein, we discuss the synthesis of isatin-1,2,3-triazole hybrid molecules *via* click chemistry and their anti-inflammatory potential in LPS-induced THP-1 cells.

Inflammation is an immunological response of the body to several factors including dietary lipids, mechanical injury, burns, allergens and noxious stimuli.[39] Usually, inflammation is a defence mechanism of the body; however, chronic inflammation may damage normal functioning, especially in the context of autoimmune diseases, atherosclerosis, arthritis, *etc.* The inflammatory response involves various immune cells, blood vessels, and molecular mediators. Key players include white blood cells (such as neutrophils, macrophages, and lymphocytes), cytokines, chemokines, and other signalling molecules. The synthesized isatin-1,2,3-triazoles **3a–i** were treated with LPS-induced human monocytic THP-1 cells *in vitro* to assess their ability to attenuate the production of three proinflammatory cytokines *i.e.*, TNF-α, IL-6 and MCP-1, and investigate the nuclear factor character of the most potent analogue in modulating the expression of concerned genes in the tested monocytic cells.

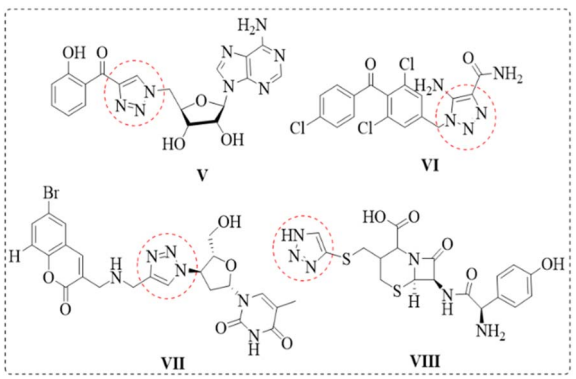


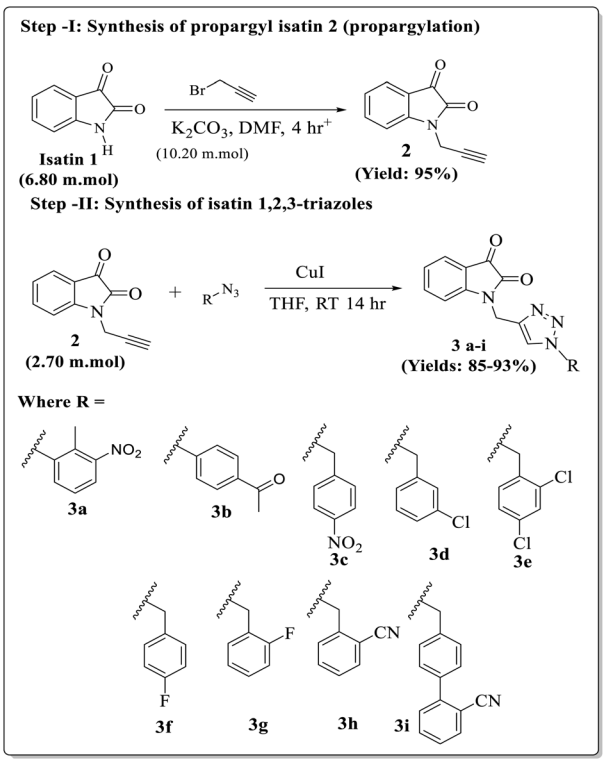
Fig. 2 Structures of some reported bioactive triazole derivatives.

2 Results and discussion

2.1 Chemistry

The synthetic strategy of novel isatin *N*-substituted 1,2,3-triazoles is outlined in Scheme 1. Isatin **1** was *N*-propargylated with propargyl bromide (1.5 eq.) in the presence of anhydrous K_2CO_3 (2 eq.) in 10 mL of DMF at room temperature for 4 h, resulting in *N*-propargyl-isatin **2**. Compound **2** was reacted with substituted phenyl azides **a** and **b** and benzyl azides **c–i** (prepared *in situ*, as previously reported[40] under click chemistry reaction conditions (Cu-catalyzed 1,3-dipolar cycloaddition)) in the presence of copper(I) iodide as the catalyst in dry THF at room temperature for 12–14 h, resulting in the production of novel substituted 1-((1-phenyl-1*H*-1,2,3-triazol-4-yl) methyl) indoline-2,3-diones **3a** and **b** (yield: 90–92%) and 1-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl) indoline-2,3-diones **3c–i** (yield: 85–93%), respectively.

All the synthesized isatin-triazole derivatives **3a–i** were characterized by ^{1}H NMR, ^{13}C NMR, IR and HR-MS spectra. In



Scheme 1 General scheme for the synthesis of isatin-1,2,3-triazol derivatives (reaction conditions: step-1: propargyl bromide (1.0 mL), K_2CO_3, DMF, RT 4 h; step-2: CuI, THF, RT, 14 h).

the IR spectra, compounds **3a–i** presented IR absorption bands ranging from 1020–1250, 1724–1740 and 3000–3134 cm^{-1} for C–N, C=O and aromatic C–H stretching, respectively. In the ^1H NMR spectra, the characteristic protons of compounds **3a** and **3e** were assigned as follows. In compound **3a**, one singlet at δ 5.57 (2H) confirmed the presence of an N–CH_2 linkage. The characteristic triazole CH proton appeared at δ 8.28 (1H) and seven aromatic protons (four aromatic protons of the isatin ring plus three aromatic protons of the phenyl ring) resonated in the range of δ 7.39–8.27. Furthermore, the structure of compound **3a** was confirmed by ESI-MS, and a molecular ion peak at m/z 386 (M + 23) was observed for the molecular formula $C_{18}H_{13}N_5O_4$ (M = 363). In the ^1H NMR spectrum of compound **3e**, the presence of two characteristic singlets at δ 5.6 (2H) to 4.9 (2H) confirmed the N–CH_2 and Ar–CH_2 linkages, respectively. A characteristic singlet peak was observed at δ 8.19 (1H) for the CH proton of the triazole ring. Furthermore, in the ^{13}C-NMR spectrum of compound **3e**, three characteristic peaks were observed for N–CH_2, Ar–CH_2 and CH of the triazole ring at δ 51.01, 35.36 and 123.1 respectively. In the ESI-mass spectrum, a molecular ion peak at m/z 409 (M + 23) was also observed for the molecular formula $C_{18}H_{12}Cl_2N_4O_2$ (M = 386); thus, the structure of **3e** was confirmed.

2.2 Anti-inflammatory activity

2.2.1 Effect of isatin-triazole conjugates on cell viability – MTT assay. The cytotoxicity of the synthesized conjugates on THP-1 cells was determined *via* the trypan blue exclusion method and further confirmed *via* MTT. The THP-1 cells were treated with compounds **3a–i** at 4 mM and 8 mM, where the parent compound isatin and the anti-inflammatory standard dexamethasone (Dex) did not have any toxic effects and the cell viability of **3a–i** was approximately equal to that of the control cells (Fig. 3 and Table 1). Hence, for subsequent experiments, the same dosage pattern was employed for each treatment.

2.2.2 Effect of isatin-triazole conjugates on the LPS-induced increase in TNF-α in THP-1 cells. In THP-1 cells, LPS has been shown to induce the production of proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and MCP-1 *via* the TLR4-NFκB signalling pathway.[41] The amount of TNF-α secreted into the cell culture supernatants was measured *via* ELISA; the data are shown in Fig. 4 and Table 2. The uninduced THP-1 cells released basal levels of TNF-α (152.70 pg mL^{-1}), and induction with LPS for 3 h increased the release of TNF-α to 53671.32 pg mL^{-1}. This LPS-dependent increase in TNF-α secretion was significantly inhibited by pretreatment with the synthesized analogues. LPS induced the

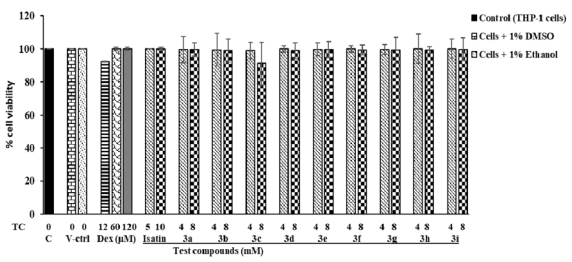


Fig. 3 MTT assay of isatin-triazole conjugates **3a–i** in THP-1 cells. The viability of the cells after treatment with 4 mM and 8 mM compounds for 16 h at 37 °C and 5% CO_2 was determined *via* the MTT assay. Samples with media alone treated with compounds were used as the blanks. The data are presented as the mean ± SD of 3 independent experiments.

Table 1 Tests of % cell viability after pretreatment with the anti-inflammatory standard compound dexamethasone (Dex), the parent compound (isatin) and its derivative compounds (**3a–i**) *via* the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay

Sample name	% of cell viability at different concentrations		
Dexamethasone (μM)			
	12	**60**	**120**
Dex	92.18% ± 0.44%	100% ± 1%	100% ± 1.02%
Isatin (mM)			
	5	**10**	
Isatin	100% ± 0.21%	100% ± 0.28%	
Isatin derivatives (mM)			
	4		**8**
3a	99.41349% ± 8%		99.40299% ± 4%
3b	99.29078% ± 10%		98.80478% ± 7%
3c	98.97% ± 5%		91% ± 13%
3d	100% ± 2%		98.69% ± 5%
3e	99.68% ± 4%		99.38% ± 5%
3f	100% ± 2%		99.30% ± 3%
3g	99.66% ± 3%		99.07% ± 8%
3h	100% ± 9%		99.33% ± 2%
3i	100% ± 6%		99.65% ± 7%

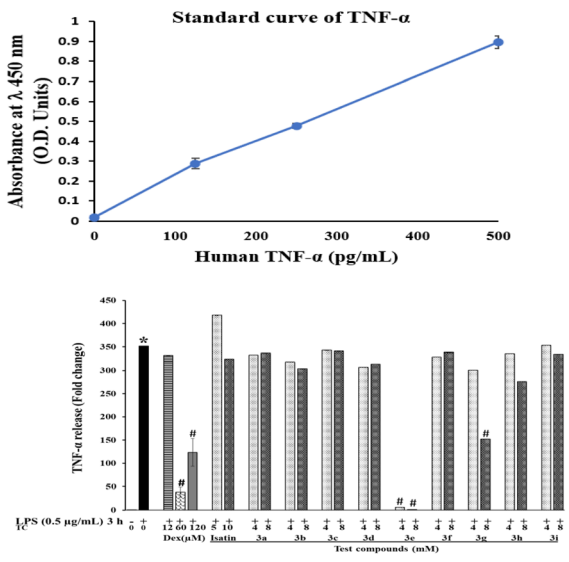


Fig. 4 Effects of the anti-inflammatory standard compound Dex, the parent compound isatin, and its derivatives **3a–i** on LPS-induced TNF- α secretion in THP-1 cells. THP-1 cells were pretreated with Dex, isatin and its derivative compounds **3a–i** for 12 h, followed by 3 h of LPS treatment. Experiments were performed at least in triplicate, and the results are expressed as mean ± S.D. *p < 0.001 for comparison between LPS-induced cells and LPS-uninduced cells. #p < 0.001 compared between cells treated with LPS in the presence of test compounds *vs.* control.

Table 2 Effects of synthesized samples on LPS-induced TNF-α secretion in THP-1 cells. THP-1 cells were pretreated with the anti-inflammatory standard compound Dex, the parent compound isatin and its derivatives for 12 h, followed by LPS for 3 h

TNF-α release (fold change)			
Cells (control)	1 ± 0.00177		
Cells + LPS (3 h)	351.46 ± 0.0248		
Pretreatment with dexamethasone (μM) for 12 h			
	12	**60**	**120**
Cells + Dex + LPS	331.6891 ± 0.05	38.21044 ± 9.97	123.3 ± 29.83
Isatin (mM)			
	5		**10**
Cells + isatin + LPS	418.10 ± 0.06		324.2066 ± 0.09
Isatin derivatives (mM)			
	4		**8**
Cells + **3a** + LPS	332.72 ± 0.01		336.55 ± 0.09
Cells + **3b** + LPS	318.06 ± 0.24		303.58 ± 0.07
Cells + **3c** + LPS	342.84 ± 0.06		341.04 ± 0.02
Cells + **3d** + LPS	306.9061 ± 0.08		313.6059 ± 0.14
Cells + **3e** + LPS	6.65 ± 0.01		1.50 ± 0.004
Cells + **3f** + LPS	328.15 ± 0.12		338.54 ± 0.05
Cells + **3g** + LPS	300.87 ± 0.06		152.19 ± 0.11
Cells + **3h** + LPS	335.6804 ± 0.11		276.31 ± 0.18
Cells + **3i** + LPS	353.60 ± 0.07		334.40 ± 0.08

secretion of TNF-α by 351.46 fold in the THP-1 cells. Among the tested samples (**3a–i**), compound **3e** significantly attenuated the LPS-induced secretion of TNF-α by 6.6 fold and 1.5 fold at all the tested concentrations of 4 mM and 8 mM, followed by **3g**, which was significant (152.1 fold) only at 8 mM. Dexamethasone (Dex) attenuated the LPS-induced secretion of TNF-α by 331.68, 38.21, and 123 fold at concentrations of 12 μM, 60 μM, and 120 μM, respectively. After 12 h of pretreatment instead of 30 min with Dex at 12 μM,[42] it did not affect the secretion of TNF-α. However, pretreatment with 60 μM Dex under the same conditions significantly attenuated the secretion of TNF-α.

2.2.3 Effect of 3e on LPS-induced IL-6 in THP-1 cells. IL-6 is a pleiotropic cytokine that initiates the acute phase response of innate immunity. The uninduced THP-1 cells released basal levels of IL-6 (96.8 pg mL^{-1}), and induction with LPS for 3 h increased the release of IL-6 to 770.5 pg mL^{-1}. LPS induced the secretion of IL-6 by 7.95 fold in the THP-1 cells. Pre-treatment of induced THP-1 cells was performed with standard dexamethasone, parent isatin or compound **3e** (given that only **3e** could significantly reduce IL-6). Pre-treatment of induced THP-1 cells with the standard dexamethasone (Dex) at three different concentrations (12 μM, 60 μM, and 120 μM) attenuated IL-6 secretion by 5.74, 6.11, and 5.53 fold, respectively. Isatin (parent compound) markedly attenuated the LPS-induced secretion of IL-6 by 4.08 fold at a concentration of 5 mM. Sample **3e** (Fig. 5 and Table 3) markedly attenuated the LPS-induced secretion of IL-6 by 1.03 fold and 1.41 fold at concentrations of 4 mM and 8 mM, respectively.

2.2.4 Effect of 3e on LPS-induced MCP-1 in THP-1 cells. Monocyte chemoattractant protein-1 (MCP-1) is a key chemokine involved in the regulation, migration and infiltration of monocytes to the site of inflammation. Uninduced THP-1 cells released basal levels of MCP-1 (204 pg mL^{-1}), and induction with LPS for 3 h increased the release of MCP-1 to 2934.6 pg mL^{-1}. In lipopolysaccharide (LPS)-induced THP-1 cells, MCP-1 production was significantly upregulated (by 14.37 fold) as

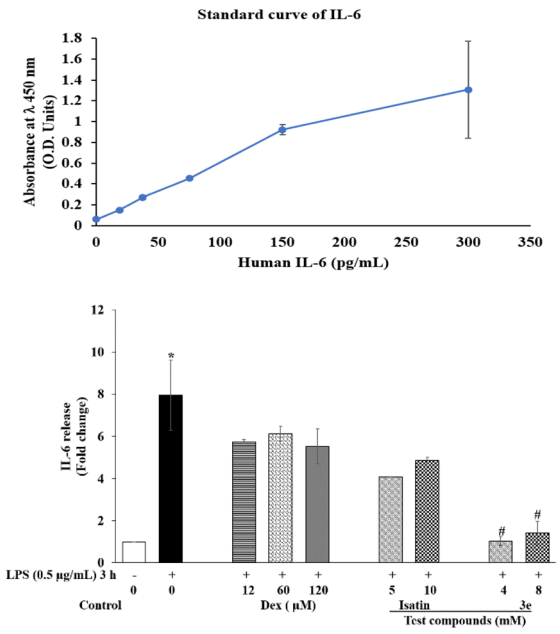


Fig. 5 Effects of the anti-inflammatory standard compound dexamethasone, the parent compound isatin, and **3e** on LPS-induced IL-6 secretion in THP-1 cells. THP-1 cells were pretreated with Dex, isatin or its derivative compound **3e** for 12 h, followed by 3 h of LPS treatment. Experiments were performed in at least triplicate, and the results are expressed as mean ± S.D. *$p < 0.001$ for comparison between LPS-induced cells and LPS-uninduced cells. #$p < 0.001$ compared between cells treated with LPS in the presence of test compounds *vs.* control.

Table 3 Effects of isatin derivative **3e** on LPS-induced IL-6 secretion in THP-1 cells. THP-1 cells were pretreated with anti-inflammatory standard compound Dex, the parent compound isatin and **3e** for 12 h, followed by LPS for 3 h

IL-6 release (fold change)			
Cells (control)	1 ± 0.0062		
Cells + LPS (3 h)	7.95 ± 1.67		
Pretreatment with dexamethasone (μM) for 12 h			
	12	**60**	**120**
Cells + Dex + LPS	5.74 ± 0.1	6.11 ± 0.36	5.53 ± 0.82
Isatin (mM)			
	5		**10**
Cells + isatin + LPS	4.08 ± 0.01		4.86 ± 0.15
3e (mM)			
	4		**8**
Cells + **3e** + LPS	1.03 ± 0.20		1.41 ± 0.53

Table 4 Effects of isatin derivative **3e** on LPS-induced MCP-1 secretion in THP-1 cells. THP-1 cells were pretreated with the anti-inflammatory standard compound Dex, the parent compound isatin and **3e** for 12 h, followed by LPS for 3 h

MCP-1 release (fold change)			
Cells (control)	1 ± 0.04		
Cells + LPS (3 h)	14.37 ± 0.12		
Pretreatment with dexamethasone (μM) for 12 h			
	12	**60**	**120**
Cells + Dex + LPS	4.93 ± 0.08	7.75 ± 1.23	7.71 ± 1.1
Isatin (mM)			
	5		**10**
Cells + isatin + LPS	10.07 ± 0.15		10.95 ± 0.097
3e (mM)			
	4		**8**
Cells + **3e** + LPS	3.32 ± 0.11		1.75 ± 0.08

part of the inflammatory response (Fig. 6 and Table 4). Treatment of these differentiated THP-1 cells with standard dexamethasone markedly attenuated MCP-1 production by 4.93, 7.75, and 7.71 fold at concentrations of 12 μM, 60 μM, and 120 μM, respectively. Similarly, compound **3e** markedly attenuated the secretion of MCP-1 by 3.32 fold and 1.75 fold at concentrations of 4 mM and 8 mM, respectively.

2.2.5 Effect of the isatin-triazole analogue 3e on TNF-α, IL-6 and MCP-1 transcript levels in LPS stimulated THP-1 cells. Gene expression regulation is a critical aspect of understanding the cellular response to various stimuli. The exposure of THP-1 monocytes to LPS strongly induced TNF-α, IL-6 and MCP-1 gene expression with maximal expression occurring after 3 h of stimulation. To assess the nuclear factor characteristic of compound **3e**, the mRNA expression levels of these cytokine genes were determined *via* qRT-PCR. Although the expression of all the above-mentioned cytokine genes was upregulated in stimulated THP-1 monocytes, the IL-6 gene presented the highest fold change in expression (6612.6), followed by the TNF-α (135.2) and MCP-1 genes (68.8).

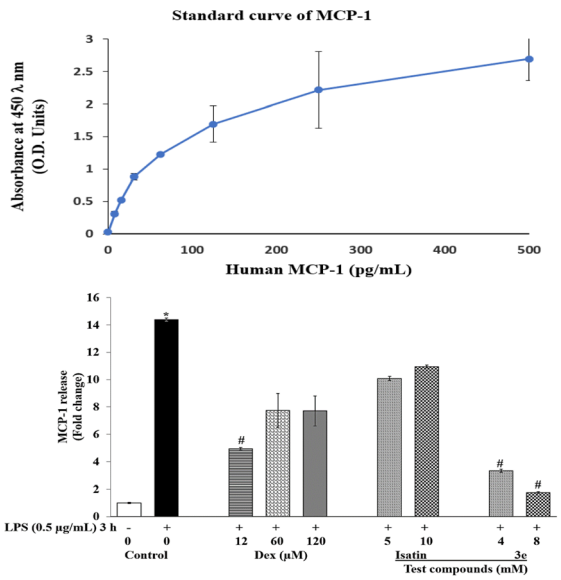


Fig. 6 Effects of the anti-inflammatory standard compound dexamethasone, the parent compound isatin, and **3e** on LPS-induced MCP-1 secretion in THP-1 cells. THP-1 cells were pretreated with Dex, isatin or its derivative compound **3e** for 12 h, followed by 3 h of LPS treatment. Experiments were performed at least in triplicate, and the results are expressed as mean ± S.D. *p < 0.001 for comparison between LPS-induced cells and LPS-uninduced cells. #p < 0.001 compared between cells treated with LPS in the presence of test compounds *vs.* control.

2.2.6 Effect of 3e on TNF-α genes. Tumour necrosis factor-alpha (TNF-α) is a proinflammatory cytokine that plays a pivotal role in inflammation and immune responses. The expression of TNF-α is tightly regulated at the transcriptional level, and its dysregulation is associated with numerous pathological conditions, including sepsis, autoimmune diseases, and cancer. Lipopolysaccharides (LPS) are known to induce the expression of TNF-α, making them a useful model for studying inflammatory responses. The gene expression regulation of TNF-α demonstrated that the TNF-α gene transcript was markedly upregulated in the LPS-treated cells compared with the uninduced control cells (135 fold). Pretreatment with isatin significantly inhibited TNF-α expression by 55.9 and 31.4 fold at concentrations of 5 mM and 10 mM, respectively. In contrast, pretreatment with sample **3e** significantly inhibited TNF-α expression by 24 and 25 fold at concentrations of 4 mM and 8 mM, respectively (Fig. 7 and Table 5).

2.2.7 Effect of 3e on IL-6 genes. Interleukin-6 (IL-6) is a cytokine that plays a vital role in the immune response, inflammation, and haematopoiesis. It is involved in various physiological processes and pathological conditions, including autoimmune diseases, chronic inflammation, and cancer. The expression of IL-6 is tightly regulated, and its dysregulation can lead to severe inflammatory responses. The gene expression regulation of IL-6 demonstrated that the IL-6 gene transcript was significantly upregulated in the LPS-treated cells compared with the uninduced control cells (6612 fold). Pretreatment with isatin

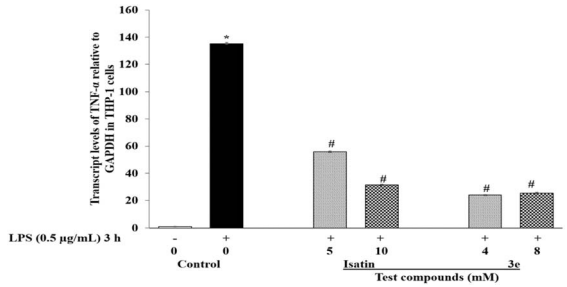


Fig. 7 Effects of the parent compound isatin and its derivative **3e** on LPS-induced TNF-α gene expression, as determined *via* quantitative real time PCR. THP-1 cells were pretreated with isatin or **3e** for 12 h, followed by 3 h of LPS induction. At the end of the treatment, the transcript levels of TNF-α were quantified *via* real-time PCR. Experiments were performed at least in triplicate, and the results were expressed as mean ± S.D. *p < 0.001 for comparison between LPS-induced cells *vs.* LPS-uninduced cells. #p < 0.001 compared between cells treated with LPS in the presence of test compounds *vs.* control.

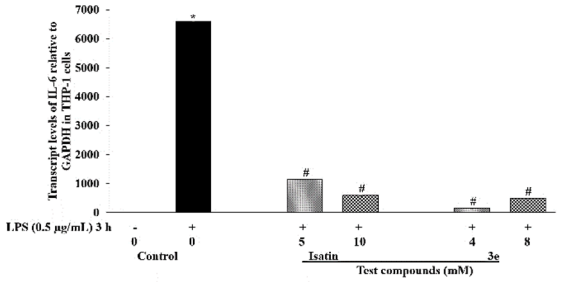


Fig. 8 Effects of the parent compound isatin and its derivative **3e** on LPS-induced IL-6 gene expression, as determined *via* quantitative real-time PCR. THP-1 cells were pretreated with isatin or **3e** for 12 h, followed by 3 h of LPS induction. At the end of the treatment, the transcript levels of IL-6 were quantified *via* real-time PCR. Experiments were performed at least in triplicate, and the results were expressed as mean ± S.D. *p < 0.001 for comparison between LPS-induced cells *vs.* LPS-uninduced cells. #p < 0.001 compared between cells treated with LPS in the presence of test compounds *vs.* control.

(parent compound) significantly inhibited IL-6 expression by 1145 and 596 fold at concentrations of 5 mM and 10 mM, respectively. In contrast, pretreatment with sample **3e** significantly inhibited IL-6 expression by 148 and 502 fold at concentrations of 4 mM and 8 mM, respectively (Fig. 8 and Table 5).

2.2.8 Effect of 3e on MCP-1 genes. Monocyte chemoattractant protein-1 (MCP-1) is a key chemokine involved in the recruitment of monocytes to sites of injury and infection. It plays a crucial role in inflammatory responses and has been implicated in various pathological conditions, including atherosclerosis, rheumatoid arthritis, and other inflammatory diseases. The regulation of MCP-1 expression is tightly controlled at the genetic level, and its overexpression can lead to excessive inflammatory responses, contributing to disease progression. The gene expression regulation of MCP-1 demonstrated that the MCP-1 gene transcript was markedly upregulated in LPS-treated cells compared with the uninduced control cells (69 fold). Pretreatment with isatin (parent compound) significantly inhibited MCP-1 expression by 6.21, and 1.73 fold at concentrations of 5 mM and 10 mM, respectively. Pretreatment with sample **3e** significantly inhibited MCP-1 expression by 50 and 25 fold at concentrations of 4 mM and 8 mM, respectively (Fig. 9).

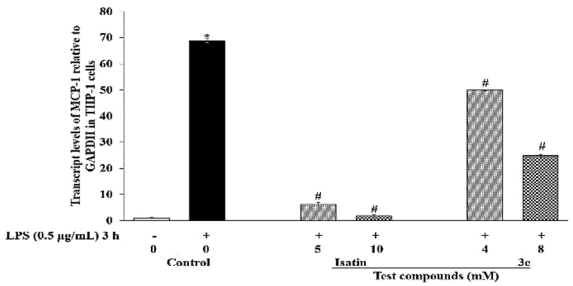


Fig. 9 Effects of the parent compound isatin and its derivative **3e** on LPS-induced MCP-1 gene expression, as determined *via* quantitative real-time PCR. THP-1 cells were pretreated with isatin or **3e** for 12 h, followed by 3 h of LPS induction. At the end of the treatment, the transcript levels of MCP-1 were quantified *via* real-time PCR. The data were presented as mean ± S.D. *p < 0.001 for comparison between LPS-induced and LPS-uninduced cells, and the experiments were conducted at least three times. #p < 0.001 when comparing cells treated with LPS and test compounds to the control.

3 Experimental

3.1 Chemistry

The melting points of all the compounds were recorded on a Casia-Siamia (VMP-AM) melting point apparatus and uncorrected. IR spectra were recorded on a PerkinElmer FT-IR 240 C spectrometer using KBr optics. NMR spectra were recorded on a Bruker Avance 400/500 MHz in DMSO-d_6 using TMS as an internal standard. All reactions were monitored on silica gel percolated TLC plates from Merck, and the spots were visualized with UV light. The silica gel (100–200 mesh) used for column chromatography was procured from Merck.

3.1.1 General experimental procedure for compound 2. Compound **1** (1.0 mmol) and potassium carbonate (1.5 mmol)

Table 5 Transcript levels of TNF- α, IL-6 and MCP-1 relative to that of GAPDH in the THP-1 cells (in fold changes)

			Cells + isatin + LPS		Cells + **3e** + LPS	
Genes	Cells (control)	Cells + LPS	5 mM	10 mM	4 mM	8 mM
TNF-α	1 ± 0.31	135.2 ± 0.81	55.9 ± 0.50	31.4 ± 0.39	23.9 ± 0.41	25.3 ± 0.59
IL-6	1 ± 0.18	6612.6 ± 0.21	1145.8 ± 1.01	596.8 ± 0.83	148.5 ± 0.53	502.5 ± 2.2
MCP-1	1 ± 0.19	68.8 ± 0.70	6.21 ± 0.67	1.73 ± 0.54	49.9 ± 0.21	24.9 ± 0.49

were dissolved in 10 mL of dry DMF and propargyl bromide (1.0 mmol) was subsequently slowly added to the mixture while stirring. The reaction mixture was stirred at room temperature for 4 h. To the reaction mixture, excess ethyl acetate was added, and the mixture was washed with water (70 mL × 3). The organic layer was dried over Na_2SO_4, filtered, concentrated under reduced pressure and washed with hexane to obtain the final analytically pure compound **2**. Red solid, yield: 95%.

*3.1.1.1 1-(Prop-2-ynyl)indoline-2,3-dione (**2**).* Orange solid, yield: 98%, m.p.156–157 °C, ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.74–6.79 (t, 1H), 6.64–6.66 (d, J = 7.4 Hz, 1H), 6.22–6.30 (m, 2H), 3.62 (d, J = 2.4 Hz, 2H), 2.24 (t, 1H). ESI-MS found MNa^+, 208 calculated for $C_{11}H_7NO_2$: M, 185.1820.

3.1.2 General experimental procedure for compounds 3a–i. Compound **2** (1.0 mmol) was dissolved in dry THF (Na 10 mL) and a catalytic amount of copper iodide was added. To this end, substituted aromatic azides (phenyl and benzyl) (1.0 mmol) in dry THF were added slowly while stirring at room temperature under a nitrogen atmosphere for 14 h. Later, excess $CHCl_3$ was added, and the mixture was filtered, and washed with $CHCl_3$. The solvent was removed under reduced pressure to obtain the crude product. The crude product was purified through silica gel (100–200 mesh) column chromatography (in ethyl acetate–hexane) to afford the desired products **3a–i**.

*3.1.2.1 1-((1-(2-Methyl-3-nitrophenyl)-1H-1,2,3-triazol-4-yl) methyl)indoline-2,3-dione (**3a**).* Orange solid, yield: 92%, m.p. 198–199 °C, IR (KBr) 3145, 3093, 1739, 1612, 1527, 1467, 1296, 1175, 1097 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 8.26–8.28 (dd, J = 8.1, 1.3 Hz, 1H), 8.12 (s, 1H), 7.85–7.90 (m, 2H), 7.79–7.81 (dd, J = 7.9, 1.3 Hz, 1H), 7.73–7.76 (t, J = 8.0 Hz, 1H), 7.61 (t, J = 7.62 Hz, 1H), 7.50 (s, 1H), 7.36–7.43 (m, 1H), 5.37 (s, 2H), 1.84 (s, 3H). HR-MS found MH^+, 364.1036. Calculated for $C_{18}H_{14}N_5O_4$: MH, 364.1046 [*m*/*z*: 364.10 (100.0%), 386.08.10 (30.2%), 146.06 (15%), 308.10 (12%), 158.06 (7%)].

*3.1.2.2 1-((1-(4-Acetylphenyl)-1H-1,2,3-triazol-4-yl)methyl) indoline-2,3-dione (**3b**).* Orange solid, yield: 90%, m.p. 252–253 °C, IR (KBr) 3153, 2959, 2924, 1737, 1687, 1608, 1468, 1263, 1158, 1098 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 8.05 (s, 1H), 7.19–7.22 (d, J = 8.7 Hz, 2H), 7.07–7.10 (d, J = 8.7 Hz, 2H), 6.65–6.71 (m, 2H), 6.21–6.28 (m, 2H), 4.15 (s, 2H), 1.68 (s, 3H). HR-MS found: MH^+, 347.1154. Calculated for $C_{19}H_{15}N_4O_3$: MH, 347.1164 [*m*/*z*: 347.11 (100.0%), 348.11 (20%), 349.12 (4%), 345.13 (2%)].

*3.1.2.3 1-((1-(4-Nitrobenzyl)-1H-1,2,3-triazol-4-yl)methyl) indoline-2,3-dione (**3c**).* Orange solid, yield: 86%, m.p. 232–234 °C, IR (KBr) 3126, 2954, 1788, 1731, 1610, 1519, 1467, 1269, 1184, 1033 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.33 (s, 1H), 7.25–7.27 (d, J = 8.7 Hz, 2H), 6.66–6.71 (t, J = 7.8 Hz, 1H), 6.60–6.62 (d, J = 7.2 Hz, 1H), 6.53–6.56 (d, J = 8.6 Hz, 2H), 6.16–6.22 (m, 2H), 4.79 (s, 2H), 4.03 (s, 2H). ESI-MS found: MNa^+, 386. Calculated for $C_{15}H_{14}N_5O_4$: MH, 364.3330 [*m*/*z*: 386 (100.0%), 235 (22.6%), 118 (10%), 166 (8%), 402 (6%)].

*3.1.2.4 1-((1-(3-Chlorobenzyl)-1H-1,2,3-triazol-4-yl)methyl) indoline-2,3-dione (**3d**).* Orange solid, yield: 89%, m.p. 145–146 °C, IR (KBr) 3130, 3069, 1732, 1609, 1468, 1322, 1217, 1124, 1049 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 9.01 (s, 1H), 8.03–8.15 (m, 4H), 7.60–7.64 (d, J = 15.8 Hz, 2H), 7.14–7.22 (m, 2H), 5.09 (s, 2H), 3.35 (s, 3H). ^{13}C-NMR ($CDCl_3$, 75 Hz) δ (ppm): 196.89, 182.97, 149.98, 143.20, 139.38, 138.09, 136.34, 130.06, 124.48, 123.40, 121.91, 119.56, 117.68, 111.07, 34.99, 26.81. HR-MS found: MH^+, 353.0813. Calculated for $C_{18}H_{14}ClN_4O_2$: MH, 353.0823 [*m*/*z*: 353.08 (100.0%), 355.07 (31.2%), 356.08 (8%), 357.08 (1.8%)].

*3.1.2.5 1-((1-(2,4-Dichlorobenzyl)-1H-1,2,3-triazol-4-yl) methyl)indoline-2,3-dione (**3e**).* Orange solid, yield: 85%, m.p. 175–176 °C, IR (KBr) 3123, 3080, 2924, 2855, 1786, 1731, 1610, 1559, 1432, 1229, 1120, 1031 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.970 (s, 2H), 5.657 (s, 2H), 7.108–7.147 (m, 2H), 7.236–7.257 (d, 1H, J = 8.533 Hz), 7.552–7.575 (dd, 1H, J = 1.506 Hz), 7.608–7.651 (m, 1H), 7.688–7.694 (d, 1H, J = 2.259 Hz), 8.199 (s, 1H). ^{13}C-NMR ($CDCl_3$, 75 Hz) δ (ppm): 183.02, 157.92, 150.18, 142.12, 138.62, 135.88, 134.38, 131.42, 130.53, 129.93, 128.02, 125.34, 124.06, 123.17, 117.51, 111.50, 51.01, 35.36. HR-MS found: MH^+, 387.0415. Found for $C_{18}H_{13}C_{12}N_4O_2$: MH, 387.0425 [*m*/*z*: 387.04 (100.0%), 389.03 (63.5%), 359.04 (7%), 331.04 (4.2%)].

*3.1.2.6 1-((1-(4-Fluorobenzyl)-1H-1,2,3-triazol-4-yl)methyl) indoline-2,3-dione (**3f**).* Orange solid, yield: 93%, m.p. 186–187 °C, IR (KBr) 3150, 2924, 1786, 1726, 1607, 1509, 1466, 1354, 1266, 1051 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.27 (s, 1H), 6.61–6.68 (m, 2H), 6.39–6.43 (dd, J = 8.6, 5.6 Hz, 2H), 6.19–6.27 (dd, J = 17.5, 8.4 Hz, 4H), 4.61 (s, 2H), 4.01 (s, 2H). HR-MS found: MH^+, 337.1114. Calculated for $C_{18}H_{14}FN_4O_2$: MH, 337.1124 [*m*/*z*: 337.11 (100.0%), 338.11 (21%), 281.11 (11.1%), 309.11 (8.6%), 282.11 (1.9%)].

*3.1.2.7 1-((1-(2-Fluorobenzyl)-1H-1,2,3-triazol-4-yl)methyl) indoline-2,3-dione (**3g**).* Orange solid, yield: 90%, m.p. 137–138 °C, IR (KBr) 3126, 3080, 2936, 1740, 1611, 1466, 1326, 1236, 1145, 1039 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.25 (s, 1H), 6.60–6.68 (dd, J = 19.1, 3.9 Hz, 2H), 6.25–6.45 (m, 4H), 6.18–6.20 (d, J = 7.8 Hz, 3H), 4.67 (s, 2H), 4.01 (s, 2H). HR-MS found: MH^+, 337.1115. Calculated for $C_{18}H_{14}FN_4O_2$: MH, 337.1125 [*m*/*z*: 337.11 (100.0%), 338.11 (21%), 309.11 (18%), 310.11 (4%)].

*3.1.2.8 2-((4-((2,3-Dioxoindolin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)methyl)benzonitrile (**3h**).* Orange solid, yield: 86%, m.p. 166–167 °C, IR (KBr) 3081, 3032, 2960, 2230, 1862, 1724, 1608, 1467, 1327, 1120, 1024 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ (ppm): 7.31 (s, 1H), 6.93–6.96 (dd, J = 7.7, 0.9 Hz, 1H), 6.6.-6.76 (m, 4H), 6.38–6.41 (d, J = 7.8 Hz, 1H), 6.18–6.22 (dd, J = 7.8, 1.6 Hz, 2H), 4.83 (s, 2H), 4.03 (s, 2H). HR-MS found: MH^+, 344.1190. Calculated for $C_{19}H_{14}N_5O_2$: MH, 344.1200 [*m*/*z*: 344.11 (100.0%), 366.09 (60%), 345.11 (38%), 288.11 (37%), 316.11 (7.1%), 289.11 (7.6%)].

*3.1.2.9 4′-((4-((2,3-Dioxoindolin-1-yl)methyl)-1H-1,2,3-triazol-1-yl)methyl)biphenyl-2-carbonitrile (**3i**).* Orange solid, yield: 85%, m.p. 170–171 °C, IR (KBr) 3143, 2950, 2221, 1854, 1738, 1612, 1469, 1327, 1122, 1098 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): δ 7.37 (s, 1H), 7.00–7.03 (d, J = 7.7 Hz, 1H), 6.83–6.86 (d, J = 6.8 Hz, 1H), 6.63–6.71 (dd, J = 15.5, 7.6 Hz, 6H), 6.47–6.50 (d, J = 8.1 Hz, 2H), 6.20–6.25 (m, 2H), 4.73 (s, 2H), 4.05 (s, 2H). HR-MS found: MH^+, 420.1482. Calculated for $C_{25}H_{18}N_5O_2$: MH, 420.1492. [*m*/*z*: 420.14 (100.0%), 421.14 (18%), 442.12 (45%), 443.13 (8%)]

3.2 Anti-inflammatory activity

3.2.1 Chemicals. The chemicals used in this study included lipopolysaccharide (LPS), trypan blue, MTT, isopropanol and chloroform from Sigma-Aldrich. ELISA kits (for human-TNF-α, IL-6 and MCP-1) were purchased from BD Biosciences (San Diego, CA, USA). The iScript cDNA synthesis kit from Bio-Rad (Hercules, CA, USA) was used. Power SYBR green PCR Master Mix was procured from Applied Biosystems (Warrington, UK). Fetal bovine serum (FBS), Pen Strep, and RPMI 1640 media were procured from Gibco (USA). All other reagents were of analytical grade.

3.2.2 Cell cultures and treatments. THP-1 cells were obtained from NCCS (Pune, India) and grown in RPMI-1640 with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) pen strep (*Penicillin streptomycin*). The cells were kept in an incubator at 37 °C and 5% CO_2.[43] The trials were carried out at a cell density of 5×10^5. The trypan vital staining exclusion technique was used to determine the viability of the cultured cells. The cells were treated with isatin triazoles (**3a–i**) at two different concentrations (4 mM and 8 mM) for anti-inflammatory drug purposes, and their viability was tested *via* the trypan vital staining exclusion method. For the trypan blue vital assay, the treated cell suspension was mixed with trypan blue solution in at a 1 : 1 ratio. Colourless and blue cells were recorded based on morphology through an inverted microscope while being watched with a haemocytometer.[44]

3.2.3 Cell viability (MTT assay). The effects of various concentrations of the selected pure compounds and isatin triazoles on THP-1 cells were tested *via* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide (MTT) assays, as described previously by Reddi & Tetali (2019).[44] THP-1 cells were distributed at a density of 5×10^5 cells/mL in 24-well plates, treated with different concentrations of test compounds and incubated at 37 °C overnight. After overnight incubation, the cells were harvested and the cell pellet was washed three times with RPMI medium. Then, 20 μL of MTT (5 mg mL^{-1}) dissolved in media was added, and the mixture was incubated for 4 h. The cells were pelleted *via* low-speed centrifugation and incubated for 15 min with 100 μL of DMSO to dissolve the insoluble purple colored formazan crystals. The absorbance of MTT formazan was determined at λ of 570 nm with the reference λ of 690 nm using a 96-well plate reader (Thermo Fisher Scientific). Incomplete media with the tested compounds were used as blanks. The percentage of viable cells from each well after incubation with the test compounds was obtained using following equation:

$$\% \text{ viability} = (\text{O.D. of treated cells//O.D. of untreated cells}) \times 100.$$

3.2.4 Treatment of Dex. The anti-inflammatory effect of Dex (0.01 μM to 1 μM) was reported in THP-1 cells,[42] and therefore we included it using higher concentrations (12 μM, 60 μM, and 120 μM) as a reference standard compound.

3.2.5 Measurement of secreted TNF-α, MCP-1, and IL-6. Secreted TNF-α, IL-6 and MCP-1 are proinflammatory cytokines and chemokines secreted by THP-1 cells and were measured with BD OptEIA-™ Set Human ELISA kits (San Diego, CA, USA). THP-1 cells at a density of 5×10^5 cells per mL were incubated with and without different concentrations of test compounds overnight and induced with LPS (0.5 μg mL^{-1}) on the following day for 3 h.[41] After induction, the cells were spun, and the supernatants were collected for quantification of the above-mentioned marker levels *via* ELISA kits (BD Biosciences). The reaction mixture was assayed by measuring the absorbance at a wavelength of 450 nm using a microplate reader with the wavelength correction set at 570 nm. The secretory levels in the culture supernatants were determined using a standard curve, which was constructed *via* serial dilutions of the above-mentioned markers, as provided with the respective kits.

3.2.6 Quantitative real time PCR. For the real-time quantitative PCR assay, two microliters of cDNA were used along with 1XFG, power SYBR green PCR Master Mix (Applied Bio Systems). By generating an amplicon melting curve by plotting fluorescence as a function of temperature, the specificity of SYBR green fluorescence was tested. The transcript levels of the marker genes were normalized to that of GAPDH mRNA.[44]

3.2.7 Quantitation of transcripts of TNF- α, IL-6 and MCP-1. Total RNA was isolated from the THP-1 cells after the indicated treatments using TRIzol reagent according to the manufacturer's (Invitrogen) protocol. cDNA was synthesized from the prepared RNA *via* an iScript™ cDNA synthesis kit. The gene expression levels of the TNF-α, MCP-1, and IL-6 genes were analysed by using 1× SYBR green PCR reagent and gene–specific primers.[43–45]

3.2.8 Statistical analysis. The entire data in the manuscript represents the mean ± standard deviation (SD) of triplicates. Data were analysed by one way analysis of variance (ANOVA).

4 Conclusion

The synthesized isatin-1,2,3-triazole hybrid analogues demonstrated significant anti-inflammatory activity, with compound **3e** showing the most potent effects. Specifically, compound **3e** significantly attenuated the secretion of TNF-α by 6.65 fold and 1.50 fold; that of IL-6 by 1.03 and 1.41 fold; and that of MCP-1 by 3.32 and 1.75 fold at concentrations of 4 mM and 8 mM, respectively. The gene expression analysis further revealed that **3e** downregulated TNF-α by 24 fold and 25 fold; IL-6 by 148 fold and 502 fold; and MCP-1 by 50 fold and 25 fold at the same concentrations. Importantly, none of the synthesized analogues, including **3e**, exhibited cytotoxicity toward THP-1 cells at the tested concentrations, confirming their safety. These findings highlight the potential of isatin-1,2,3-triazole hybrids, particularly compound **3e**, as nontoxic and effective anti-inflammatory agents.

Abbreviations

C	Control
V-ctrl	Vehicle control
LPS	Lipopolysaccharides

TNF-α	Tumor necrosis factor-alpha
IL-6	Interleukin-6
MCP-1	Monocyte chemoattractant protein-1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide
Dex	Dexamethasone
TC	Test compound

Data availability

The data supporting the findings of this study are available from the corresponding author JKK upon reasonable request.

Author contributions

A. N. K. performed the synthetic protocol and wrote the first draft of the manuscript; J. K. K. and K. V. N. S. S. edited the manuscript and formatted the last version; S. R. D. and S. D. T. performed the anti-inflammatory activity.

Conflicts of interest

Authors declare no conflict of interest.

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