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Synthesis and biological evaluation of nitrogen-containing benzophenone analogues as TNF-α and IL-6 inhibitors with antioxidant activity

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ABSTRACT

A series of nitrogen-containing benzophenone analogues were synthesized by Mannich reaction and evaluated for the inhibition of pro-inflammatory cytokines, TNF-α and IL-6. DPPH (1,1-diphenyl-2-picryl hydrazine) radical scavenging activity and its kinetics were studied to determine the antioxidant potential of the test samples. All the synthesized compounds exhibited promising activity against IL-6 in a range of 81–89%, 09–42% at 10 and 1 μM, respectively, concentration. Exceptionally, the compound **20e** was observed to be an effective inhibitor of TNF-α (54%) and IL-6 (97%), (47%) at 10 and 1 μM concentrations with minimum toxicity (22%) against CCK-8 cells. With few exceptions, all other compounds were found to be excellent inhibitors of IL-6 and moderate to excellent inhibitors of TNF-α, however the toxicity profiles of these compounds need to be ameliorated in further optimization studies. Amongst the tested compounds, **16a**, **17g**, **18f**, **18g**, **19g** and **20e** were found to possess significant antioxidant activity.

Inflammation is a multi-step and complex biological reaction involving variety of pro-inflammatory cellular proteins, enzymes and cytokines. The pro-inflammatory cytokines, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) are implicated in the pathogenesis of various inflammatory disorders such as rheumatoid arthritis (RA), inflammatory bowel disease, osteoarthritis, psoriasis, endotoxemia and/or toxic shock syndrome.[1–9] Apart from pro-inflammatory attributes these cytokines have a wide array of functions for maintaining the normal cellular physiology. For example, TNF-α can induce apoptosis and secretion of cytokines such as IL-1, IL-6 and IL-10; it can also activate T cells and other inflammatory cells. However, an overabundance of TNF-α and IL-6 is attributed to the development of various human ailments including inflammatory disorders. Targeting the inhibition of cytokines, in particular TNF-α, has been successful in several clinical trials for the treatment of RA. Nevertheless, the TNF-α inhibition has been identified as one of the attractive targets for the design and development of anti-inflammatory agents.[10]

Upregulation of tumour necrosis factor-alpha (TNF-α) has several concerns for the onset and progression of a number of diseases such as diabetes, multiple sclerosis, RA, psoriatic arthritis, cachexia, sepsis, tumourigenesis and inflammatory bowel diseases.[11] Therapies that have successfully targeted TNF-α in man include biologics directed towards these cytokines such as adalimumab (Humira), etanercept (Enbrel) and infliximab (Remicade) which have demonstrated a significant efficacy in the treatment and management of RA.[12] However, these agents require administration via injection or infusion, therefore identification of an orally available small molecule therapy would provide an additional benefit to the patients. In spite of enormous efforts, no small molecule has yet been approved to specifically inhibit TNF-α activity. Therefore, there is a continuing interest in the search of small molecules that can block TNF-α signalling without side effects and general disadvantages associated with protein drugs.

Amongst the pro-inflammatory cytokines, the IL-6 is a pleiotropic cytokine, that is, abundant in both the synovium and serum of RA patients and induces a broad range of cellular and physiological responses during the inflammation reaction. In addition to playing a role in inflammation and hematopoiesis, it is involved in other processes such as neuronal differentiation and bone loss. IL-6 is produced at the site of inflammation and plays a key role in acute phase response. IL-6 is a central regulator of inflammatory diseases, including end-stage renal disease and rheumatoid arthritis.[13] Till-date, designing the IL-6 inhibitory agents has remained a significant hope in the mainstream of anti-inflammatory drug development.

Benzophenones, the precursors for the synthesis of the title compounds are essential due to their diverse biological and chemical properties. In recent years a vast body of literature has

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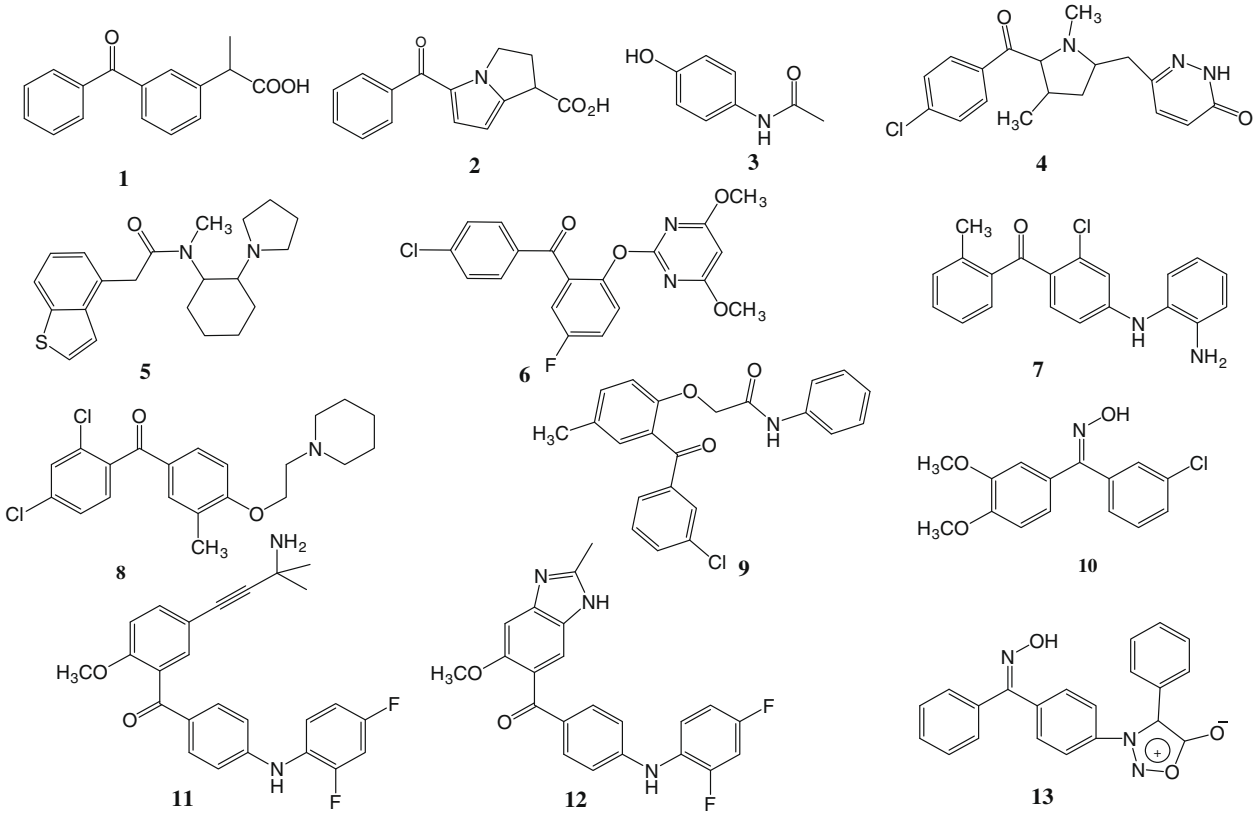


Figure 1. Some biologically active benzophenones containing nitrogen analogues.

accumulated, linking the therapeutic applications of benzophenones. The suitability of benzophenone analogues (Fig. 1) (**1**) as chemotherapeutic agents, especially as anti-inflammatory, is well cited.[14,15] Several research groups have synthesized benzophenone analogues (**2**, **3**, **4**, **5**) and reported as potent anti-inflammatory agents.[16–18] Venu et al. reported benzophenone pyrimidine analogue (**6**) as an anti-inflammatory agent.[19] Ottosen et al. have reported aminobenzophenones, which are novel class of p38 MAP kinase inhibitors (**7**) having high anti-inflammatory activity.[20] Khanum et al. identified benzophenone *N*-ethyl piperidine ether analogues[21] (**8**) and benzophenone[22] analogues (**9**) as anti-inflammatory agents. Murari et al. reported benzophenone oxime analogues (**10**) as inhibitors of phospholipase A_2 with anti-inflammatory activity.[23] Revesz et al. described benzoylpyridines and benzophenones (**11**, **12**) as p38α MAP kinase inhibitors with oral activity.[24] Compound **13** was reported as an inhibitor of secretory phospholipase A_2 with anti-inflammatory activity.[25] Based on these interesting biological activity profiles of benzophenone analogues, we inspired and made an attempt to synthesize a series of nitrogen-containing benzophenone analogues as inhibitors of TNF-α and IL-6 and as antioxidant agents.

Chemicals were purchased from Aldrich Chemical Co. USA. TLC was performed on an aluminium-backed silica plate with visualization by UV-light. Melting points were determined with a digital thermometer. IR spectra were recorded on a FT-IR Shimadzu 8300 spectrophotometer and ^{1}H NMR spectra were recorded on a Bruker 300 MHz spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard and chemical shifts are reported in δ units. Mass spectra were obtained with a Shimadzu LCMS-2010EV.

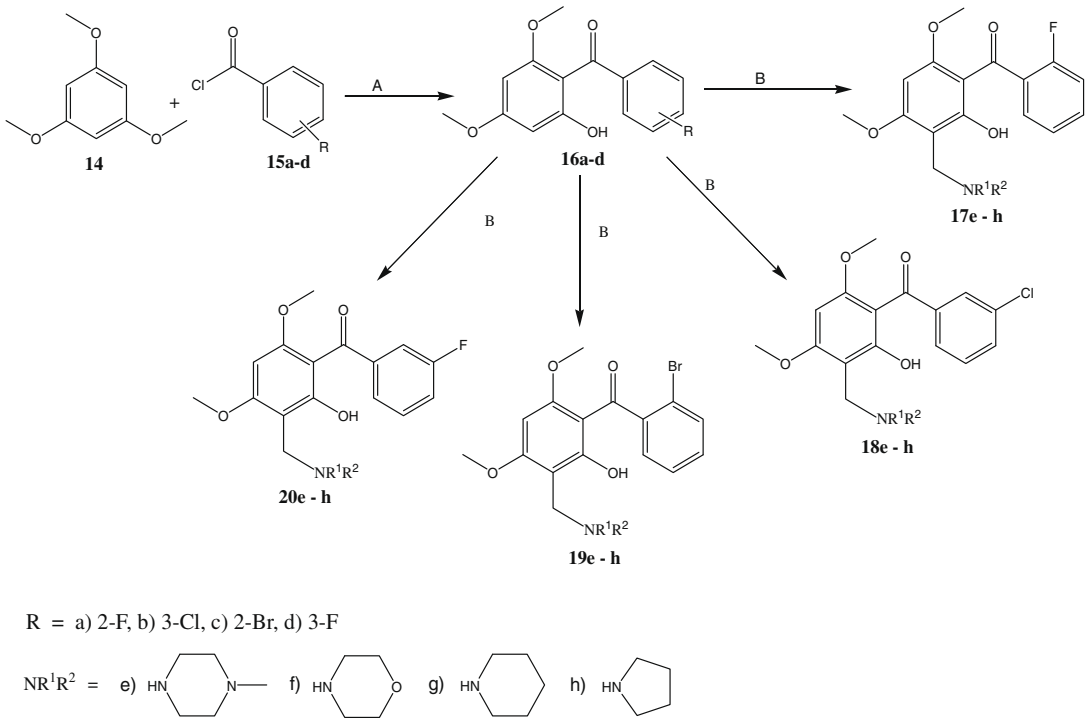
Nitrogen-containing benzophenones **16a–b**, **17e–h**, **18e–h**, **19e–h** and **20e–h** were synthesized as shown in Scheme 1. 2-Hydroxy-4,6-dimethoxybenzophenones **16a–b** were synthesized following the method described in the literature with minor modifications.[26] Condensation of **16a–b** with various secondary amines and formaldehyde in the presence of hydrochloric acid in isopropanol furnished desired derivatives **17e–h**, **18e–h**, **19e–h** and **20e–h**.[27]

The synthesized compounds **16a–b**[28], **17e–h**, **18e–h**, **19e–h** and **20e–h**[29] were characterized by IR, ^{1}H NMR and mass spectrophotometer.

The synthesized nitrogen-containing benzophenone was assayed for its biological activities against the pro-inflammatory cytokines TNF-α and IL-6[30] and antioxidant activity.[31] The DPPH radical scavenging activity and its kinetics were performed in order to determine the antioxidant potential.[32] DPPH is a stable nitrogen-centred free radical. Its reaction rates correlate directly with antioxidant activity; the higher the rate, the more effective the antioxidant.[33] The summary of reduction of DPPH (%) is shown in Table 1, while the results of the kinetics (%) of DPPH radical scavenging activity are summarized in Table 2.

Result and discussion: All the synthesized compounds were evaluated as inhibitors of TNF-α and IL-6 at 10 μM concentration. Percent inhibition activity of TNF-α and IL-6 is summarized in Table 1 at 10 μM concentration. Dexamethasone (1 μM) was used as a reference compound. Benzophenone analogues **16a–d** have shown inhibition of TNF-α in a range of 11–47%, at 10 μM. Nitrogen-containing benzophenone analogues **17e–h**, **18e–h**, **19e–h** and **20e–h** exhibited 20–100% inhibition of TNF-α at 10 μM. Amongst the series **18e**, **19f**, **19g**, **19h** and **20e** were found to be good to excellent TNF-α inhibitors.

Interestingly, all the synthesized benzophenones were observed to be promising leads, possessing excellent IL-6 inhibitory activity as compared to Dexamethasone. Benzophenone analogues **16a–d** showed 71–81% and 09–40% inhibition of IL-6 at 10 and 1.0 μM, respectively, concentration. Compounds **17e–h**, **18e–h**, **19e–h** and **20e–h** showed 83–100% and 09–57% excellent inhibition of



Scheme 1. Reagents and conditions: (A) anhyd $AlCl_3$, dry ether, 0 °C, rt to 48 h, 60%; (B) secondary amine, 37% HCHO, isopropanol, HCl reflux, 50–60%.

IL-6 at 10 and 1.0 μM, respectively, concentration. Mostly, all compounds exhibited toxicity in a range of 34–49% at 10 μM, except compounds **16a**, **18e**, **19f**, **19g** and **19h** and were found to be highly active and toxic (53–84% at 10 μM) in nature.

While discussing the structure–activity relationship, the 3-Cl and 3-F substitution with *N*-methyl piperazine/pyrrolidine (**18e**, **20e**, **20h**, **19g**, **19h**, etc.) seems to be compatible for the inhibition of IL-6 (10 μM). To explain in detail the structure–activity relationships, we have evaluated the activity at 1 μM concentration and the results are shown in Table 1. From the results it clearly indicated that substitution at 3′ position (3-Cl, 33–54% and 3-F, 30–47%) is more favourable for the inhibition of Il-6, while substitution at 2′ position (2-F, 15–21% and 2-Br, 9–22%) is not favourable for the inhibition of IL-6, however the same cannot hold true for other compounds demonstrating significant IL-6 inhibition. It has been also observed that the toxicity concerns were found to be more extreme with 3-Cl and 2-Br substitutions as compared to those with fluoro substitution at 2′ and 3′. It is indeed difficult to attribute the structure–activity

Table 1
TNF-α and IL-6 inhibitory activities (%) and DPPH (%) radical scavenging activity of N-containing benzophenone analogues

Entry	TNF-α	IL-6		Toxicity	DPPH (%)
		10 μM	1 μM		
16a	47	71	11	53	32.35
16b	00	78	31	49	16.45
16c	11	72	07	43	NR
16d	00	81	38	24	NR
17e	19	83	21	40	17.53
17f	27	89	19	39	19.68
17g	00	84	18	31	73.31
17h	10	87	15	27	24.80
18e	100	100	54	77	NR
18f	01	83	42	39	33.43
18g	11	83	33	31	45.29
18h	37	81	41	53	5.13
19e	20	81	09	23	13.48
19f	65	97	19	66	6.23
19g	78	98	22	61	64.43
19h	99	100	19	84	NR
20e	54	97	47	22	40.44
20f	21	86	35	42	NR
20g	00	87	30	27	24.70
20h	01	93	34	34	7.29
DMS (1 μM)	73	84		00	00
GA (1 mM)	ND	ND	ND	ND	90.83

The results summarized are the mean values of $n = 2$, DMS = dexamethasone, GA = gallic acid, ND = not determined, NR = not reactive.

Table 2
Kinetics of the DPPH radical scavenging activity (%) of N-containing benzophenone analogues at 1 μM concentration

Entry	DPPH (%) Time in min				
	2	4	6	8	10
16a	8.20	12.52	13.55	16.83	18.86
16b	3.21	3.90	4.20	6.52	7.60
16c	NR	NR	NR	NR	NR
16d	NR	NR	NR	NR	NR
17e	3.30	3.95	4.00	5.40	6.20
17f	3.50	4.20	4.74	6.80	8.21
17g	13.68	16.88	22.98	28.92	34.64
17h	3.60	4.24	6.45	9.50	11.90
18e	NR	NR	NR	NR	NR
18f	5.52	6.44	9.45	14.56	15.94
18g	9.20	11.40	14.80	20.95	22.82
18h	0.5	1.60	1.82	2.10	2.11
19e	3.20	3.95	4.50	6.80	7.59
19f	0.8	1.20	1.90	2.11	2.95
19g	10.80	14.15	17.70	25.45	32.50
19h	NR	NR	NR	NR	NR
20e	9.50	15.80	18.50	19.45	22.58
20f	NR	NR	NR	NR	NR
20g	4.20	5.98	9.25	11.75	13.66
20h	0.92	4.51	2.11	3.78	4.78
GA (1 μM)	18.23	22.68	19.45	38.42	46.90

The results summarized are the mean values of $n = 2$, NR = no reaction, GA = gallic acid.

relationship in the case of TNF-α inhibition because the compounds with (**18g**, **20g**) and without (**16b**, **16d**) *N*-methyl substitution were found to be nonreactive with TNF-α. In general it can be summarized that the effective inhibition of TNF-α, IL-6 and severe toxicity seem to be closely related with 3-Cl and 2-Br N-methylated analogues as compared to 2-F and 3-F analogues.

The antioxidant potentials have been described to be closely associated with anti-inflammatory activities. In general pharmacological notion, compounds possessing reducing abilities can be a potential candidate for the inhibition of cyclooxygenase, a key enzyme implicated in inflammation.[34] However, in the present studies a paradoxical relation was observed with the compounds (**18e**, **19h**) possessing significant TNF-α and IL-6 inhibitory activities but nonreactive with DPPH. It was also noted that the condensation of piperidine (**17g**, **18g** and **19g**) was found to be more favourable for the manifestation of DPPH radical scavenging activities. All other compounds showed moderate DPPH reducing potentials. The kinetic study also revealed that the compounds **17g**, **18g**, **19g** and **20e** were observed to be more reactive towards DPPH as compared to all other compounds under study.

In conclusion, a new series of nitrogen-containing benzophenone analogues were synthesized and evaluated for inhibition of TNF-α and IL-6 along with antioxidant activity. The result of the present investigations indicates the importance of these new compounds as potential candidates of anti-inflammatory and antioxidant agents. From the activity results of the tested compounds, **20e** showed promising activity against TNF-α and IL-6 with less cytotoxicity and considerable antioxidant activity. The results of the other compounds as inhibitors of TNF-α and IL-6 are certainly encouraging, but the cytotoxicity of these compounds limits the therapeutic applications. However, the basic nitrogen-containing benzophenone moiety can be considered as one of the scaffolds for the design and development of anti-inflammatory agents targeting TNF-α and IL-6. The importance of the compound **20e** cannot be ignored as it is one of the intermediates in the lead optimization process.

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28. *Compound* **16a***:* Faint yellow compound, mp 122–124 °C: IR: 1622; ^{1}H NMR (300 MHz, $CDCl_3$): 13.08 (s, 1H), 7.401 (t, 1H, *J* = 5.8 Hz), 7.372 (t, 1H, *J* = 5.8 Hz), 7.185 (d, 1H, *J* = 4 Hz), 7.02 (d, 1H), 6.12 (d, 1H), 5.86 (d, 1H), 3.84 (s, 3H), 3.44 (s, 3H), MS: 277 [M+1]. *Compound* **16b***:* Faint yellow compound, mp 118–120 °C: IR: 1625; ^{1}H NMR (300 MHz, $CDCl_3$): 12.24 (s, 1H), 7.491–7.351 (m, 4H), 6.17 (d, 1H), 5.93 δ (d, 1H), 3.86 (s, 3H), 3.48 (s, 3H), MS: 293 [M+1]. *Compound* **16c***:* Faint yellow compound, mp 138–140 °C: IR: 1634; ^{1}H NMR (300 MHz, $CDCl_3$): 13.38 (s, 1H), 7.590 (d, 1H, *J* = 8 Hz), 7.390 (d, 1H, *J* = 8 Hz), 7.352 (m, 1H), 7.23 (t, 1H, *J* = 6 Hz, 4 Hz), 6.17 (d, 1H), 5.86 (d, 1H), 3.91 (s, 3H), 3.45 (s, 3H), MS: 337 [M+1]. *Compound* **16d***:* Off white compound, mp 78–80 °C: IR: 1635; ^{1}H NMR (300 MHz, $CDCl_3$): 12.83 (s, 1H), 7.531–7.395 (m, 4H), 6.13 (d, 1H), 5.84 (d, 1H), 3.89 (s, 3H), 3.77 (s, 3H), MS: 277 [M+1].
29. *Compound* **17e***:* Off white compound, mp 142–144 °C: IR: 1618; ^{1}H NMR (300 MHz, $CDCl_3$): 7.695 (dd, 1H, *J* = 8 Hz), 7.441(m, 1H), 7.185 (dd, 1H, *J* = 8 Hz, 7 Hz), 7.035 (m, 1H), 5.971 (s, 1H), 3.840 (s, 3H), 3.720 (s, 2H), 3.645 (s, 3H), 2.595–2.455 (m, 8H), 2.260 (s, 3H), MS: 389 [M+1]. *Compound* **17h***:* Off white compound, mp 146–148 °C: IR: 1652, 1615; ^{1}H N MR (300 MHz, $CDCl_3$): 8.325 (d, 1H, *J* = 8 Hz), 7.684 (t, 1H, *J* = 8 Hz, 6 Hz), 7.421 (d, 1H, *J* = 6 Hz), 7.377 (t, 1H, *J* = 6 Hz, 8 Hz), 6.41 (s, 1H), 4.14 (s, 3H), 3.99 (s, 2H), 3.97 (s, 3H), 2.644 (s, 4H), 1.75 (s, 4H), MS: 360 [M+1]. *Compound* **18e***:* Yellow sticky compound, IR: 1619; ^{1}H NMR (300 MHz, $CDCl_3$):7.421 (d, 1H, *J* = 8 Hz), 7.381–7.315 (m, 3H), 5.92 (s, 1H), 3.860 (s, 3H), 3.713 (s, 3H), 3.615 (s, 3H), 2.635–2.417 (m, 8H), 2.33 (s, 3H), MS: 405 [M+1]. *Compound* **18f***:* White Compound, mp 120–122 °C; IR 1679, 1634; ^{1}H NMR (300 MHz, $CDCl_3$): 7.795 (d, 1H, *J* = 2 Hz), 7.775 (t, 1H, *J* = 6 Hz), 7.496 (t, 1H, *J* = 4 Hz), 7.377 (d, 1H, *J* = 8 Hz), 6.05 (s, 1H), 3.86 (s, 3H), 3.71 (s, 2H), 3,71 (s, 3H), 3.69 (t, 4H), 2.59 (t, 4H), MS: 372 [M+1]. *Compound* **19e***:* Off white compound, mp 78–80 °C; IR 1618; ^{1}H NMR (400 MHz, $CDCl_3$): 7.395 (d, 1H, *J* = 8 Hz), 7.382–7.25 (m, 3H), 5.89 (s, 1H), 3.87 (s, 3H), 3.695 (s, 2H), 3.585 (s, 3H), 2.61–2.45 (m, 8H), 2.29 (s, 3H), MS: 450 [M+1]. *Compound* **19f***:* Yellow compound, mp132–134 °C; IR: 1619; ^{1}H NMR (400 MHz, $CDCl_3$): 7.550 (d, 1H, *J* = 8 Hz), 7.335 (d, 1H, *J* = 6 Hz), 7.230 (d, 1H, *J* = 8 Hz), 7.19 (d, 1H, *J* = 8 Hz), 5.89(s, 1H), 3.88 (s, 3H), 3.72 (t, 4H), 3.66 (s, 2H), 3.48 (s, 3H), 2.57 (t, 4H), MS: 437 [M+1]. *Compound* **19g***:*Off white compound, mp 109–111 °C IR: 1618; ^{1}H NMR (400 MHz, $CDCl_3$): 7. 552 (d, 1H, *J* = 8 Hz), 7.395 (d, 1H, *J* = 7.6 Hz), 7.319 (t, 1H, *J* = 7.2 Hz, 7.6 Hz), 7.220 (t, 1H, *J* = 7.2 Hz, 8 Hz), 5.908 (s, 1H), 3.842 (s, 3H), 3.649 (s, 2H), 3.557 (s, 3H), 2.509 (s, 4H), 1.578 (t, 6H), MS: 435 [M+1]. *Compound* **19h***:* Yellow compound, mp 79–81 °C; IR: 1617; ^{1}H NMR (400 MHz, $CDCl_3$): 7.552 (d, 1H, *J* = 8 Hz), 7.312 (t, 1H, *J* = 7.6 Hz, 4.8 Hz), 7.216 (m, 2H), 5.887 (s, 1H), 3.863 (s, 3H), 3.759 (s, 2H), 3.504 (s, 3H), 2.627 (s, 4H), 1.779 (s, 4H), MS: 420 [M+1]. *Compound* **20e***:*Yellow compound, IR: 1613; ^{1}H NMR (300 MHz, $CDCl_3$): 7.527(d, 1H, *J* = 8 Hz), 7.338 (d, 1H, *J* = 7.6 Hz) 7.289 (t, 1H, *J* = 7.6 Hz, 2.4 Hz), 7.227 (m, 1H), 5.86 (s, 1H), 3.84 (s, 3H), 3,67 (s, 2H), 3.48 (s, 3H), 2.60 (s, 4H), 2.46 (s, 2H), 2.25 (s, 3H), MS: 389 [M+1]. *Compound* **20f***:* Yellow compound, IR: 1619; ^{1}H NMR (40 MHz, $CDCl_3$): 7.664 (d, 1H, *J* = 8 Hz), 7.541 (d, 1H, *J* = 9.6 Hz), 7.396 (m, 1H), 7.226 (ddd, 1H, *J* = 2.4 Hz, 2.4 Hz, 2.8 Hz), 6.055 (s, 1H), 3.860 (s, 3H), 3.769 (s, 2H), 3.710 (s, 3H), 3.695 (s, 4H), 2.588 (s, 4H), MS: 376 [M+1].
30. *Assay for TNF-α and IL-6 inhibition:* Pro-inflammatory cytokine production by lipopolysaccharide (LPS) in THP-1 cells was measured according to the method described by Hwang et al., 1933.[35] Briefly, THP-1 cells were cultured in RPMI 1640 culture medium (Gibco BRL, Pasley, UK) containing 100 U/ml penicillin and 100 mg/ml streptomycin (100× solution, Sigma Chemical Co. St. Louis, MO) containing 10% foetal bovine serum (FBS, JRH). The cells were differentiated with phorbol myristate acetate (PMA, Sigma). Following cell plating, the test compounds or vehicle (0.5% DMSO) was added to each well and the plate was incubated for 30 min at 37 °C. Finally, LPS (Escherichia coli0127:B8, Sigma Chemical Co., St. Louis, MO) was added, at a final concentration of 1 μg/ml. The plates were incubated at 37 °C for 24 h, 5% CO_2. Supernatants were harvested and assayed for TNF-α and IL-6 by ELISA as described by the manufacturer (BD Biosciences). The cells were simultaneously evaluated for cytotoxicity[36] using CCK-8 from Dojindo Laboratories. Percent inhibition of cytokine release compared to that of the control was calculated.
31. *DPPH radical scavenging assay:* The DPPH radical scavenging assay was performed as described by Bartolome.[32] The reaction mixture contained 1 mM concentration of individual test sample (in absolute ethanol) and

DPPH radical (10^{-4} M in absolute ethanol) solution. The contents of the reaction mixture were observed spectrophotometrically at 517 nm after 20 min. Gallic acid was used a reference drug (90.30%).
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