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ORIGINAL RESEARCH

Inhibitory effects of α, β-unsaturated carbonyl-based compounds and their pyrazoline derivatives on the phagocytosis of human neutrophils

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Abstract

A series of α, β-unsaturated carbonyl-based compounds (curcumin analogs and chalcone derivatives) and their pyrazoline derivatives were investigated for their in vitro inhibitory effects on the phagocytosis of human neutrophils. The effects of the compounds on the chemotactic migration, CD11a/18 expression, phagocytic activity, and reactive oxygen species (ROS) production by human whole blood cells (WBC) and isolated human polymorphonuclear neutrophils (PMNs) were assessed by using 24-well cell migration assay kit, flow cytometer, Phagotest assay kit, and luminol/lucigenin-based chemiluminescence assay, respectively. Compounds **4**, **5**, **6**, **13**, **23**, **33**, **39**, and **41** showed strong inhibitory activity against PMNs chemotaxis with IC_{50} values, ranging from 0.22–1.68 μM which were much lower than those of ibuprofen and curcumin (IC_{50} values 11.02 and 5.0 μM, respectively). All compounds showed low or moderate inhibition of cell adhesion molecule expression except for compound **15**, while compounds **4**, **5**, **8**, and **21** at 100, 25, and 6.25 μg/mL showed strong inhibition of opsonized bacteria engulfment by neutrophils with the highest suppressive effects exhibited by compound **21** at 37.4%. Compounds **4**, **11**, **13**, **14**, **24**, **25**, **27**, **33**, and **34** significantly suppressed ROS generation by PMNs and WBC. Compounds with N-methyl 4-piperidone and 4-piperidone linkers (**4**, **13**, **14**, **23**, **24**) and 2-pyrazoline-1-carboxamide and 2-pyrazoline-1-carbothioamide derivatives showed strong inhibition on chemotactic and phagocytic activities, and ROS production. The results suggest that some of the α, β-unsaturated carbonyl-based compounds strongly modulated the innate immune responses of phagocytes at different steps, highlighting their potential as a source of new immunomodulatory agents.

Keywords α, β-Unsaturated carbonyl-based compounds · Immunosuppressive effect · Chemotaxis · Phagocytosis · Reactive oxygen species

Introduction

Polymorphonuclear neutrophils (PMNs) are professional phagocytes playing important roles in our innate immune defense against pathogens such as bacteria (Casulli et al. 2014). Normally, in a healthy person, neutrophils prevail as resting state in blood circulation, which prevents the accidental release of toxic intracellular granules. However, certain agents i.e., bacterial products, chemokines, and cytokines can cause the activation of neutrophils which then migrate towards the inflamed tissue and come across activating signals to elicit bacterial killing (Ahmad et al. 2014). Upon activation, the movement of neutrophils from the circulation to the site of infection is the first step of phagocytosis (Muller 2013). The surface receptors of neutrophils i.e., L-selectins make loose connections with the

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vascular endothelium which induce the conformational changes in integrin adhesion molecules (CD11b/CD18) that further get tangled with intercellular adhesion molecule (ICAM-1 and 2), vascular cell-adhesion molecule-1 (VCAM-1), eventually resulting in strong adherence (Schmidt et al. 2013). After neutrophils have interacted with the endothelial cells they move along a chemotactic gradient to the site of infection. Neutrophils at the injured tissue phagocytose the infecting pathogens and this engulfment activity is initiated when complement surface receptors on PMNs detect complement-opsonized microbes (Haugland et al. 2012) ultimately resulting in oxygen burst and release of toxic granules for the prompt killing of bacteria (Yuanda and Husain 2017). Although this process is imperative in protecting the body from the infectious microbes, yet sometimes, it becomes lethal in case of inappropriate activation of neutrophils, hence incongruous release of ROS and other toxic molecules can cause harm to the host tissue, ultimately resulting in the pathology of various immune-related disorders.

The α,β-unsaturated carbonyl-based compounds are considered to be the reactive substructures of natural products or synthetic molecules, hence, owing miscellaneous pharmacological activities i.e., potent antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, anti-tubercular, and immunomodulatory properties. In the α,β-unsaturated carbonyl-based compounds, the carbon-carbon double bond and carbon-oxygen double bond are segregated by only one carbon-carbon single bond i.e., double bonds are conjugated. These conjugations not only give the properties of the functional group attached but also give some other properties to the compounds. The most important and widely studied α, β-unsaturated carbonyl-based compounds include the natural products, curcumin, chalcones and their analogs and derivatives (Arshad et al. 2017). In fact one-sixth of the currently known natural products contain these potentially reactive substructures (Rodrigues et al. 2016). Curcumin, extracted from the rhizomes of *Curcuma longa* is a hydrophobic polyphenol as well as the active constituent of turmeric found to exhibit a wide range of activities (Anand et al. 2008) while flavonoids, either isolated from natural sources or chemically synthesized, have also acquired continuous importance due to their ample biological activities (Elias et al. 1999; Ni et al. 2004; Bukhari et al. 2013). Previously, we synthesized different novel series of α, β-unsaturated carbonyl-based compounds. The first series of compounds (**1**–**30**) were investigated as acetylcholinesterase and butyrylcholinesterase inhibitors (Bukhari et al. 2014a) along with the inhibition of enzymes involved in arachidonic acid metabolism pathway, and it was documented that these compounds have been found to be strong inhibitors of aforementioned enzymes (Bukhari et al. 2014b). Other compounds (**31**–**41**) were investigated for their inhibitory effects on human cancer cell lines and on the diphenolase activity of mushroom tyrosinase and it was found that some of the compounds exhibited highly potent anticancer and tyrosinase inhibiting activities (Qin et al. 2015). Besides, this series of compounds has also been evaluated against the sPLA2-V, COX-1, COX-2, and release of pro-inflammatory cytokines including IL-6 and TNF-α (Bukhari et al. 2015a).

These findings encouraged us to evaluate the effects of these compounds on the innate immune system and hence explored the relationship between the inhibitory effects and chemical structures of the compounds. Therefore, in this study we investigated the in vitro inhibitory effects of this series of α, β-unsaturated carbonyl-based compounds along with their pyrazoline derivatives on the successive steps of phagocytosis. The phagocytosis steps include chemotaxis of PMNs, expression of adhesion molecule CD11b/CD18, phagocytic activity and inhibition of intracellular and extracellular reactive oxygen species (ROS) production by PMNs and human whole blood.

Methods and materials

General method for the synthesis of α, β-Unsaturated carbonyl-based compounds

A novel series of α, β-unsaturated carbonyl-based compounds have been synthesized previously by our group using Claisen-Schmidt condensation reaction. The first series of compounds (twenty one compounds, **1**–**7, 11**–**17, 21**–**27**) was prepared by condensation between different ketones and suitable aryl aldehydes at a molar ratio 1: 2 in the presence of NaOH in ethanol. Compounds (**8**–**10, 18**–**20, 28**–**30**) resembling chalcone analogs were synthesized by same Claisen Schmidt condensation using molar ratio 1:1 of ketone and aldehyde (Fig. 1) (Bukhari et al. 2014b). Other compounds related to chalcone analogs were synthesized by substituting thioacetamide and arylacetamide groups at their N1 position in three different series *i.e.*, chalcones (**31**–**34**) in which equimolar ketones and aldehydes were reacted in the presence of a base. Further, these chalcones (**31**–**34**) and thiosemicarbazide were refluxed at alkaline pH to synthesize pyrazolines (**35**–**37**). A similar reaction was used to yield compounds (**38**–**41**) by refluxing chalcones (**31**–**34**) with *N*-(4-chlorophenyl) semicarbazide in alkaline conditions (Fig. 2) (Qin et al. 2015). Curcumin, ibuprofen and aspirin were used as reference compounds in this study based on their role in inflammation and that they have been used as positive controls in similar studies (Jantan et al. 2011; Bukhari et al. 2015b).

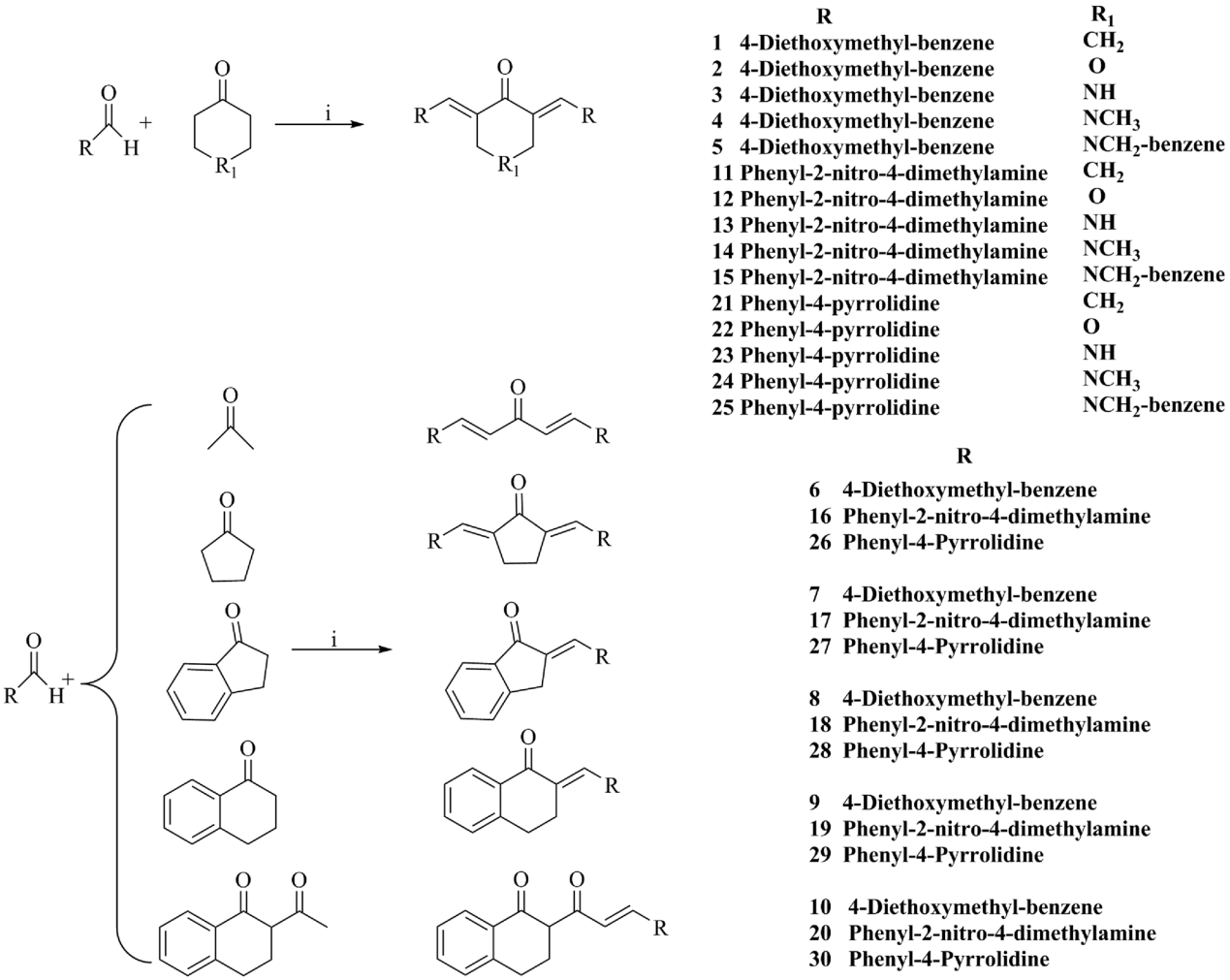

Fig. 1 General synthesis of α, β-unsaturated carbonyl-based compounds. Reprinted from the European Journal of Medicinal Chemistry (Bukhari et al. 2014a)

Chemicals and reagents

Serum opsonized zymosan A (*Saccharomyces cerevisiae* suspensions and serum), luminol (3-aminophthalhydrazide), phorbol 12-myristate 13-acetate (PMA), lucigenin (10, 10'-dimethyl-9, 9'-biacridinium, dinitrate), acetyl salicylic acid (purity 99%) and ibuprofen (purity 99%) were purchased from Nacalai Tesque, Japan. Chemiluminescence measurements were carried out on a Luminoskan Ascent luminometer (Thermo Scientific, UK). Cytoselect 24-well cell migration assay kits, Roswell Park Memorial Institute (RPMI)-1640, fetal bovine serum (FBS) penicillin, streptomycin were obtained from Cell Bio labs, Inc. (San Diego, CA, USA). A CO_2 incubator from Shell Lab, USA, and the inverted microscope was purchased from Olympus ck 30, Tokyo, Japan. Phagotest kit was obtained from Glycotope Technology, Germany and allophycocyanin (APC)-conjugated CD11b, FITC-conjugated CD18, and IgG-FITC were obtained from BD Biosciences (Franklin Lakes, NJ, USA). The flow cytometer BD FACS Canto II equipped with 488 nm argonion laser was used. Lymphoprep™ gradient (1077 mg/mL), trypan blue reagent, phosphate buffer saline tablet (PBS), Hanks Balance Salt Solutions (HBSS), and dimethylsulfoxide (DMSO) were purchased from Sigma (St Louis, MO, USA).

Isolation of human polymorphonuclear neutrophils (PMNs)

The isolation of PMNs from the whole blood from healthy human volunteers aged ≥18 years old was performed by following the method described by Koko et al. with slight modification (Koko et al. 2008). The volunteers fulfilled the following inclusion criteria; fasted overnight and did not take any supplements or medicine and non-smoker. Briefly, 10 mL of fresh blood collected from the volunteers was

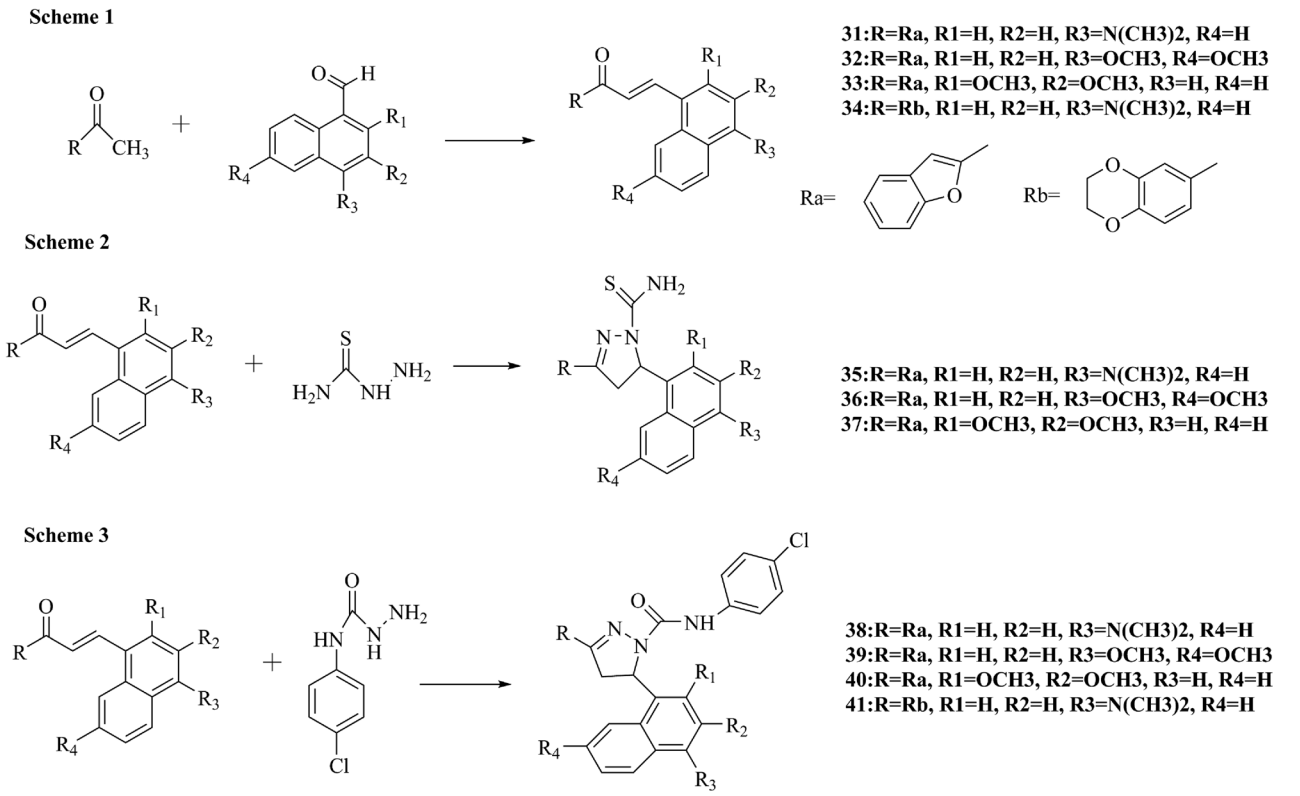

Fig. 2 Structures and synthesis of chalcone derivatives (scheme 1) and pyrazoline derivatives (scheme 2, 3). Reprinted from the Chemical Biology and Drug Design (Bukhari et al. 2015a, 2015b)

allowed to sediment for 30 min. After the specified time interval, the separated layer of plasma was carefully layered into a 10 mL falcon tube containing 5 mL of lymphoprep gradient (1077 mg/mL) without any break to the gradient. The Falcon tube, with two-step gradients of lymphoprep and blood was centrifuged at 400×*g* for 20 min. The neutrophils and erythrocytes sedimented through the lymphoprep layer during centrifugation. For the lysis of RBCs, 1 mL of distilled water was added followed by the addition of HBSS and centrifuged at 300×*g* for 5 min. The supernatant was aspirated carefully without disturbing the pellet and resuspended in HBSS. Finally, the cell suspension was diluted with HBSS to obtain a final cell suspension of 1×10^6/mL. The use of human blood was approved by the Ethics Committee of the Universiti Kebangsaan Malaysia (approval no. FF/2012/Ibrahim/23May/432-May2012-August 2013).

Cell viability

Trypan blue exclusion assay was employed to assess the viability of cells. The synthetic compounds at four different doses i.e., 200, 100, 50, and 6.25 µg/mL were incubated with PMNs (1×10^6/mL) in 5% CO_2 incubator, each in triplicate, at 37 °C for 2 h at room temperature. The cells with the blue dye uptake were considered as dead cells and the percentage cell viability was calculated from the total cell counts (Jantan et al. 2012).

Cell migration assay

The inhibitory effect of the compounds on neutrophils migration was quantitatively measured by using modified method of Ilangkovan et al. (2015). The neutrophils isolated from the heparinized whole blood were adjusted to a final concentration of 1.5×10^6 cells. Cytoselect 24-well cell migration assay kit was used to perform the experiment. Initially, a total of 500 µL RPMI media comprising of chemotactic stimulant, 10% FBS was added to the lower chambers of the 24-well plate while the cell suspension, with 1.5×10^6 cells in 300 µL serum-free media and serial dilutions of tested sample (10, 5, 2.5, 1.25 µg/mL) was added to the upper chamber (consisting of a polycarbonate membrane insert, with 3 µm pore-sized filters) of a 24-well tissue culture plate. Afterwards, the plate was incubated for 2.5 h at 37 °C in 5% CO_2 incubator for the cells to migrate towards the chemoattractant. The migratory cells passed through polycarbonate membrane and adhered to the bottom side. After the specified time interval, the inserts were shifted to the new wells containing cell detachment solution

and again incubated at 37 °C (5% CO_2) for 30 min to remove the cells from the bottom of inserts. Finally, the inserts were discarded and dislodged cells were stained by adding the lysis buffer/CyQuant® GR dye solution. The control wells contained RPMI with 10% FBS and cells but no compound. Ibuprofen and curcumin were used as positive controls. The number of cells migrating to lower chambers was determined by fluorescence, per manufacturer's instructions, on a Victor 2 plate reader (Perkin Elmer, Inc.).

Determination of MAC-1 expression

The Mac-1 expression assay was carried out by following the method of Harun et al. (2015) with slight modification. Aliquots (100 µL) of heparinized whole blood were incubated with 20 µL of tested samples at three different concentrations (100, 25, and 6.25 µg/mL) in 5% CO_2 incubator at 37 °C for 30 min. After half an hour, cells were stimulated with LPS at a concentration of 0.25 µg/mL and incubated for 90 min. Thereafter the tubes were simultaneously placed on ice in order to stop the reaction. Next, 10 µL of APC-conjugated CD11b and FITC-conjugated CD18 were added to the mixture. Meanwhile, 10 µL of IgG-FITC was added as a negative control and tubes were incubated on ice for 60 min. After that, FACS lysing solution was added to each tube and incubated in dark for 20 min at 37 °C to lyse the red blood cells. Subsequently, the tubes were centrifuged at a speed of 250×*g* for 10 min and the supernatant was discarded while retaining the pellet. Finally, the cells within the pellet were washed twice with PBS at the same speed. The inhibition of adhesion molecule expression was analyzed by flow cytometer. The specific mean fluorescence intensity for cells stained with each antibody was reported as a percentage of CD11b and CD18 expression. For each sample, 10,000 cells were analyzed.

Determination of phagocytic activity

The phagocytic activity assay was determined by using the Phagotest assay kit, as previously described (Ilangkovan et al. 2013). Briefly, a total of 100 µL heparinized blood was mixed with 20 µL of test samples at two different concentrations (100, 25, and 6.25 µg/mL) and incubated in a closed shaking water bath at 37 °C with 60 r.p.m. speeds for 30 min. The negative control tube without test sample was kept on ice. After the incubation, the tubes were immediately transferred to the icebox and 20 µL of fluorescent-labeled *Escherichia coli* (*E. coli*) was added into each tube which was then incubated in shaking water bath at 37 °C for 10 min, while for negative control, the samples remained on ice. Thereafter, the samples were immediately transferred on ice to stop the process of phagocytosis followed by the addition of quenching solution which suppressed fluorescence of the bacteria to bind to the outside of the cell. The sample tubes were vortexed well and centrifuged at 250×*g* for 5 min (4 °C) after adding 3 mL of washing solution. After washing twice, 2 mL of lysing solution was added and incubated in dark for 20 min at 37 °C. At the end of incubation, the samples were again washed and centrifuged at the same speed. Finally, 200 µL of DNA staining solution was added to each tube and vortexed. The phagocytic activity was analyzed by flow cytometer and was determined as the percentage of phagocytizing neutrophils. Live populations were gated by the software program in the scatter diagram (FCS versus SSC).

Chemiluminescence assay

Luminol and lucigenin based chemiluminescence assay were performed as previously described by Bukhari et al. (2015a, 2015b). Luminol and lucigenin served as a probe for the detection of intracellular and extracellular ROS production. Briefly 25 µL of whole blood diluted with sterile PBS (1:50; pH 7.4) and 25 µL of PMNs (with a final concentration of 1×10^6 cells/mL) suspended in HBSS was mixed with 25 µL of synthetic analogs dissolved in DMSO and distilled water at a ratio of 5:95. The final concentration of DMSO in the reaction mixture was maintained at 0.5% to eliminate the effect of the solvent on the chemiluminescence while the final concentrations of the synthetic compounds were 12.5, 6.25 3.125, 1.56, and 0.78 µg/mL. The reaction mixture was incubated at room temperature for 30 min. At the end of incubation, the cells were activated by 25 µL of opsonized zymosan while for extracellular ROS production, phorbol 12-myristate 13-acetate (PMA) was used to stimulate PMNs, followed by the addition of 25 µL of luminol and lucigenin, respectively. Thereafter, the final volume of each well in 96-well plate was adjusted to 200 µL by adding HBSS and incubated in the thermostatically controlled chamber of the luminometer for 50 min at 37 °C. However, the control wells contained no compound but 0.5% DMSO, HBSS, luminol, and cells. Ibuprofen and curcumin were used as positive controls. The luminometer results were monitored as the chemiluminescence reading per luminometer unit (RLU) with peak and total integral values set with repeated scans at 30 s intervals and 1 s points measuring time. The inhibition percentage (%) for each compound was calculated using the following formula:

$$\text{Inhibition}\,(\%) = \frac{\left(\text{RLU}_{\text{control}} - \text{RLU}_{\text{sample}}\right) \times 100\%}{\text{RLU}_{\text{control}}}$$

Statistical analysis

All the data were analyzed by using the Statistical Package for Social Sciences (SPSS). Each sample was measured in triplicate and the data are presented as a mean ± standard error of mean (SEM). To determine the IC_{50} values of active compounds, Graph Pad Prism 5 Software was used and the values were obtained from at least three determinations. Data were analyzed using a one-way analysis of variance (ANOVA) for multiple comparisons, and $P < 0.05$ was considered to be statistically significant.

Results and discussion

All the synthetic compounds evaluated for cytotoxicity on PMNs and human whole blood cells were found safe at three different doses i.e., 100, 50, and 6.25 µg/mL, showing percent cell viability ≥90% after 2 h of incubation. However, at 200 µg/mL, the cell viability was less than 50%. Thus, the safe concentrations used in this study were below 100 µg/mL. The results of trypan blue exclusion method indicated that the compounds were not toxic to immune cells and could possibly modify the cellular immune responses.

Effect of α, β unsaturated carbonyl based compounds on PMN chemotaxis

The effects of the forty-one α, β-unsaturated carbonyl-based compounds were studied at a single dose of 10 µg/mL on the migration of PMNs towards the chemoattractant (10% FBS). Chemoattractant buffer (DMSO and RPMI, 1:1 ratio) was used as a negative control. Compounds **3–6**, **8**, **11**, **13**, **15**, **16**, **23**, **24**, **31–33, 36–39**, **41** showed significant blockade on the directional movement with percentage inhibition ranging from 59 to 91% (Table 1). These compounds were further studied at serial dilutions of 10 to 1.25 µg/mL and compared with the positive controls, ibuprofen and curcumin. As shown in Fig. 3, all the selected samples tested followed a concentration-dependent manner of inhibition while compounds **4**, **5**, **6**, **13**, **23 33**, **39**, and **41** were found to be highly effective, with IC_{50} values, ranging from 0.22–1.68 µM which were much lower than those of the positive controls, ibuprofen and curcumin (IC_{50} values of 11.02 and 5.0 µM, respectively)(Table 1). Earlier, Bukhari et al. suggested that compounds **4** and **23** were potent anti-inflammatory agents (Bukhari et al. 2014a, 2014b). Other compounds listed in Table 1 were also more active or comparable to ibuprofen as they showed IC_{50} values ranging from 2.29 to 10.8 µM.

Table 1 Percentage inhibition at 10 µg/mL and IC_{50} values (µM) of α, β-unsaturated carbonyl based compounds and pyrazoline derivatives on activities of PMN cell movement

Compounds	% inhibition of chemotactic activity	IC_{50} values (µM)
3	61 ± 3.4*	8.79 ± 1.6
4	91 ± 2.0**	1.10 ± 1.1
5	91 ± 2.3**	0.22 ± 2.0
6	90 ± 2.0**	1.11 ± 1.7
8	73 ± 1.4*	6.83 ± 1.4
11	75 ± 2.0*	3.19 ± 1.2
13	75 ± 3.1*	1.46 ± 2.4
15	89 ± 1.4**	2.29 ± 1.8
16	65 ± 2.0*	10.8 ± 2.1
23	85 ± 3.0**	1.12 ± 1.1
24	75 ± 1.4*	4.35 ± 1.4
31	90 ± 1.2**	6.13 ± 1.6
32	87 ± 0.8*	5.66 ± 1.4
33	88 ± 1.7*	1.68 ± 1.5
36	65 ± 1.7*	8.87 ± 2.1
37	85 ± 1.7*	4.20 ± 1.4
38	59 ± 1.4*	7.78 ± 1.5
39	83 ± 2.0**	0.58 ± 0.8
41	80 ± 2.3*	0.25 ± 1.6
Ibuprofen	76 ± 7.0	11.02 ± 3.1
Curcumin	79 ± 0.8	5.0 ± 0.6

Values are presented as mean ± SD ($n = 3$). (–) IC_{50} values were not determined. Significance of differences with respect to control: $^{**}P < 0.01$; $^{*}P < 0.05$

Effect of α, β unsaturated carbonyl based compounds on MAC-1 expression

The effect of α, β-unsaturated carbonyl-based compounds on MAC-1 expression was measured by a flow cytometer. Table 2 shows the percentage of CD 11b/18 expression activity of neutrophils at 100.0, 25, and 6.25 µg/mL. Among all the α, β unsaturated carbonyl based compounds, curcumin analogs exhibited weak to mild inhibition on CD 11b/18 expression compared with the untreated sample as a positive control. On the other hand, compounds synthesized in three different schemes (chalcone and pyrazoline derivatives) did not show any inhibitory effects on Mac-1 expression (Data are not shown). Of all the compounds tested, compound **15** showed the most active inhibitory effects on CD 11b/18 expression activity with percentage expression of 40.27% at 100 µg/mL compared to the positive control. Compounds **8**, **16**, **23**, and **27** also exhibited moderate inhibition of CD 11b/18 expression at 100 µg/mL i.e., 72.93 ± 1.58–78.23% (Table 2). The inhibition of this cell adhesion by the compounds might be correlated with the blockade of various intracellular and extracellular

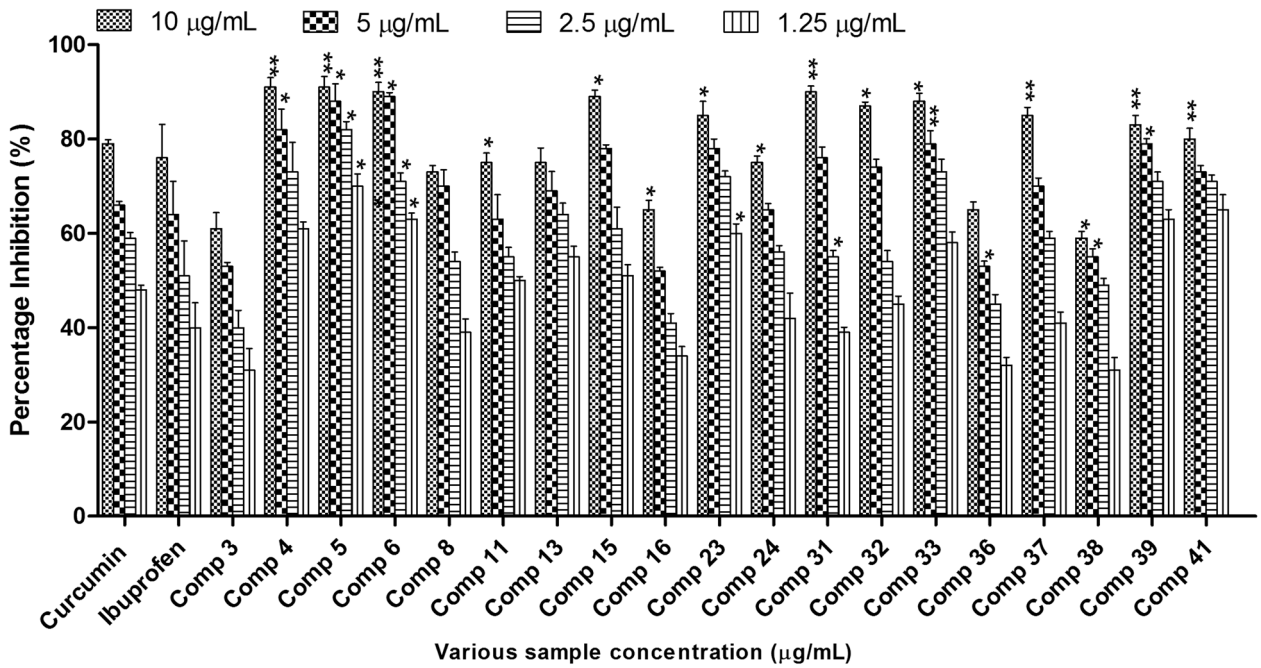

Fig. 3 Inhibitory effects of α, β-unsaturated carbonyl-based compounds and pyrazoline derivatives at 10, 5, 2.5, 1.25 μg/mL on activities of PMN cell movement. Significance of differences with respect to control: $^{**}P < 0.01$, $^{*}P < 0.05$

mechanisms particularly inhibition of NFκB pathway activation. Recently, Zhao et al. reported that monocarbonyl analog with a 2-nitro substitution strongly suppressed TNF-α which was responsible for activating NFκB pathway (Zhang et al. 2014). Also in another study, it was reported that compounds with thiopyrone structure and 4-piperidone derivative strongly inhibited cytokines release and NFκB inhibition (Zhao et al. 2015).

Effect of α, β-unsaturated carbonyl-based compounds on phagocytic activity

The ability of PMNs to phagocytize opsonized bacteria was determined by Phagotest kit and analyzed by flow cytometry. The phagocytic inhibitory activity at 0 °C was used as a negative control while at normal condition (37 °C) as a positive control, and the phagocytic activity was calculated by comparing the percentage of phagocytosis with the decline in the percentage of *E. coli* ingestion by phagocytic cells. The phagocytic activity of the cells when mixed with the α, β-unsaturated carbonyl-based compounds was less than the positive control, signifying that the compounds reduced the percentage of FITC-labeled *E. coli* intake and thus inhibiting the phagocytic cells. Notably, the phagocytic inhibitory activity of PMNs by the compounds was dose-dependent however; more pronounced effects were observed at higher doses. As shown in Table 3, compounds **4**, **5**, **8**, and **21** at 100, 25, and 6.25 μg/mL showed strong inhibition of bacteria engulfment by neutrophils with the highest suppressive effects exhibited by compound **21** i.e., 37.4%. Chalcones synthesized by three different schemes also showed good inhibition of bacteria engulfment by neutrophils ranging from 72.4 to 76.5%. Figure 4 shows flow cytometer representation of *E. coli* engulfment by neutrophils; inhibitory effects of α, β-unsaturated carbonyl-based compounds at 100 and 6.25 μg/mL on PMN phagocytic activity. CR1, CR2, and CR3, complement surface receptors on neutrophils immediately recognize complement-opsonized bacteria while FcαR, FcεRI, FcγRI receptors of PMNs, specific for the Fc region of antibody, identify antibody-coated microbes (Shokunbi and Odetola 2008). The results indicate that the enhanced inhibition of ingestion of immunoglobulins and complement opsonized *E. coli* by the tested samples might be due to the blockade of aforementioned significant receptors.

Effect of α, β-unsaturated carbonyl-based compounds on ROS production

The compounds were initially investigated on the human whole blood for the purpose of preliminary screening and it was seen that all compounds exhibited significant dose-dependent inhibition of ROS generation during the metabolic phase of phagocytosis *i.e.* increase in the concentration of the tested samples led to increase in percentage inhibition (Table 4). Both intracellular and extracellular ROS production in the whole blood was strongly inhibited by curcumin analogs **3**, **5**, and **23** with IC_{50} values of 5.84,

Table 2 Percentage of Cd11b/Cd18 expression activity of neutrophils at 100, 25, and 6.25 μg/mL of α, β-unsaturated carbonyl based compounds

Compounds	PMN Mac-1 expression activity ± SEM (%)		
	100 μg/mL	25 μg/mL	6.25 μg/mL
1	97.7 ± 0.53	97.94 ± 0.28	98.27 ± 0.44
2	94.33 ± 0.50	95.04 ± 1.15	96.47 ± 0.69
3	90.07 ± 1.75*	93.38 ± 1.58	96.57 ± 1.18
4	84.13 ± 2.47*	89.28 ± 1.50*	95.50 ± 1.80
5	87.17 ± 4.35*	91.28 ± 1.66	93.23 ± 2.29
6	92.33 ± 0.90	94.04 ± 1.49	97.07 ± 0.26
7	88.77 ± 0.75**	90.61 ± 1.12	91.53 ± 0.52
8	77.10 ± 1.27**	85.28 ± 1.04*	96.00 ± 0.50
9	81.50 ± 1.29*	86.94 ± 0.94*	90.20 ± 1.15
10	91.50 ± 0.64*	94.38 ± 1.18	96.47 ± 0.72
11	81.50 ± 0.64*	88.28 ± 0.51*	94.37 ± 1.66
12	91.13 ± 0.66	94.04 ± 0.93	96.60 ± 0.67
13	87.73 ± 1.42*	90.94 ± 0.81*	94.87 ± 1.55
14	81.30 ± 0.76	86.61 ± 0.70	94.7 ± 1.50
15	40.27 ± 1.39**	66.90 ± 1.98*	96.10 ± 0.29
16	72.93 ± 1.58**	79.55 ± 2.09*	95.57 ± 0.20
17	92.13 ± 0.64*	94.71 ± 0.42	95.27 ± 0.52
18	91.43 ± 1.21*	93.71 ± 1.25	95.17 ± 1.39
19	92.50 ± 0.44*	93.04 ± 0.96	94.37 ± 0.69
20	87.40 ± 0.69*	90.94 ± 1.78	92.03 ± 1.01
21	89.53 ± 0.41*	94.38 ± 0.61	96.33 ± 1.26
22	81.93 ± 1.92*	86.40 ± 1.29*	95.00 ± 1.93
23	78.23 ± 0.41**	83.40 ± 0.56*	92.00 ± 1.08
24	82.50 ± 1.21*	88.28 ± 0.51*	93.97 ± 0.81
25	86.60 ± 1.10*	91.61 ± 1.12	93.50 ± 0.76
26	92.57 ± 0.67*	95.38 ± 0.48	97.23 ± 0.45
27	76.57 ± 2.32**	83.41 ± 0.57*	90.67 ± 1.76*
28	89.20 ± 0.52*	89.79 ± 0.28*	90.73 ± 0.52*
29	85.67 ± 1.86*	90.61 ± 1.12	93.60 ± 1.04
30	94.57 ± 0.66	94.71 ± 0.84	96.03 ± 0.93
	Positive control	99.00 ± 0.60	

Values are presented as mean ± SEM ($n = 3$). Significance of differences with respect to control: $^{**}P < 0.01$; $^{*}P < 0.05$

3.13, and 5.92 μM, respectively, suggesting that they were highly potent. Compounds **4, 11, 13, 14, 18, 24**, and **25** also displayed high inhibitory effects with IC_{50} values ranging from 3.97–5.14 μM for luminol-enhanced ROS production while 7.24–20.42 μM for lucigenin amplified ROS generation as compared to aspirin (15.24 and 43.6 μM, respectively) and curcumin (7.4 and 14.1 μM, respectively). On the other hand, compounds **8, 9, 14, 16, 17, 18, 21, 22** and **29** with IC_{50} values ranging from 3.84–7.33 μM and 7.24–33.92 μM exhibited the good inhibitory activity of both intracellular and extracellular reactive oxygen species

Table 3 Percentage of PMN phagocytic activity at 100, 25, and 6.25 μg/mL of α, β-unsaturated carbonyl based compounds and pyrazoline derivatives

Compounds	PMN Phagocytic activity ± SEM (%)		
	100 μg/mL	25 μg/mL	6.25 μg/mL
1	62.20 ± 1.01**	69.27 ± 2.25*	74.83 ± 1.41*
2	73.56 ± 1.73**	74.13 ± 1.05*	75.50 ± 1.43*
3	62.40 ± 1.44*	68.13 ± 2.01*	73.13 ± 2.24*
4	49.37 ± 0.93**	53.67 ± 4.67*	58.03 ± 1.16*
5	43.73 ± 1.23**	55.37 ± 4.12*	60.87 ± 0.66*
6	58.43 ± 1.07**	62.57 ± 3.24*	66.13 ± 0.96*
7	70.41 ± 1.62*	72.47 ± 2.48*	78.59 ± 1.59*
8	49.73 ± 1.34**	55.37 ± 4.12*	70.60 ± 1.42*
9	53.00 ± 1.46**	62.91 ± 2.92*	69.93 ± 0.27*
10	63.10 ± 0.81*	68.50 ± 2.21*	71.80 ± 0.90*
11	62.13 ± 1.22*	64.11 ± 2.53*	65.30 ± 1.44*
12	66.57 ± 1.56**	68.39 ± 2.11*	72.93 ± 0.26*
13	57.73 ± 2.28*	61.45 ± 4.28*	63.63 ± 1.46*
14	53.70 ± 1.63*	57.38 ± 3.40**	60.30 ± 0.87*
15	57.00 ± 1.85*	63.17 ± 3.11*	67.80 ± 1.91*
16	51.00 ± 2.02**	58.52 ± 1.74*	67.67 ± 1.54*
17	70.67 ± 0.74*	74.13 ± 1.05*	76.10 ± 0.67*
18	69.80 ± 0.74*	71.80 ± 1.93*	74.83 ± 0.67*
19	67.73 ± 0.38**	70.27 ± 1.88*	74.73 ± 0.74*
20	70.53 ± 1.70**	72.13 ± 1.62*	75.62 ± 1.70*
21	37.47 ± 1.05**	57.73 ± 1.86**	63.63 ± 1.49*
22	62.17 ± 1.16*	65.11 ± 1.71*	68.10 ± 1.46*
23	67.8 ± 1.18**	70.93 ± 1.34*	74.10 ± 1.81*
24	68.37 ± 2.47*	77.13 ± 0.75	79.17 ± 1.08*
25	64.77 ± 1.03**	69.39 ± 1.18*	76.43 ± 1.81*
26	64.63 ± 1.15*	68.06 ± 0.90*	71.73 ± 0.66*
27	58.67 ± 0.95**	70.27 ± 1.30*	76.50 ± 1.75*
28	60.03 ± 0.98*	64.78 ± 1.39*	74.37 ± 1.47*
29	70.37 ± 0.61*	73.13 ± 0.98*	77.43 ± 0.93*
30	79.23 ± 1.65*	86.28 ± 2.09	91.2 ± 0.82
31	73.5 ± 0.93*	78.13 ± 1.14*	81.6 ± 1.16
32	76.4 ± 1.23*	77.47 ± 0.48*	78.7 ± 0.66*
33	76 ± 1.07*	78.47 ± 0.75*	81.1 ± 0.96
34	74.4 ± 1.62*	76.47 ± 0.55*	79.7 ± 1.59*
35	73 ± 1.34*	77.94 ± 1.89*	82.7 ± 1.42
36	72.7 ± 1.46**	75.80 ± 0.72	81.4 ± 0.27
37	73.6 ± 0.81*	76.13 ± 1.05*	80.8 ± 0.90
38	75.5 ± 1.22*	79.81 ± 1.77*	85 ± 1.44
39	72.4 ± 1.56**	74.47 ± 0.48*	80.7 ± 0.26
40	75.6 ± 2.28*	76.47 ± 0.55*	78.3 ± 1.46*
41	74.0 ± 1.63*	75.47 ± 1.43*	78.3 ± 0.87*
	Positive control	94.97 ± 0.99	

Values are presented as mean ± SEM ($n = 3$). Significance of differences with respect to control: $^{**}P < 0.01$; $^{*}P < 0.05$

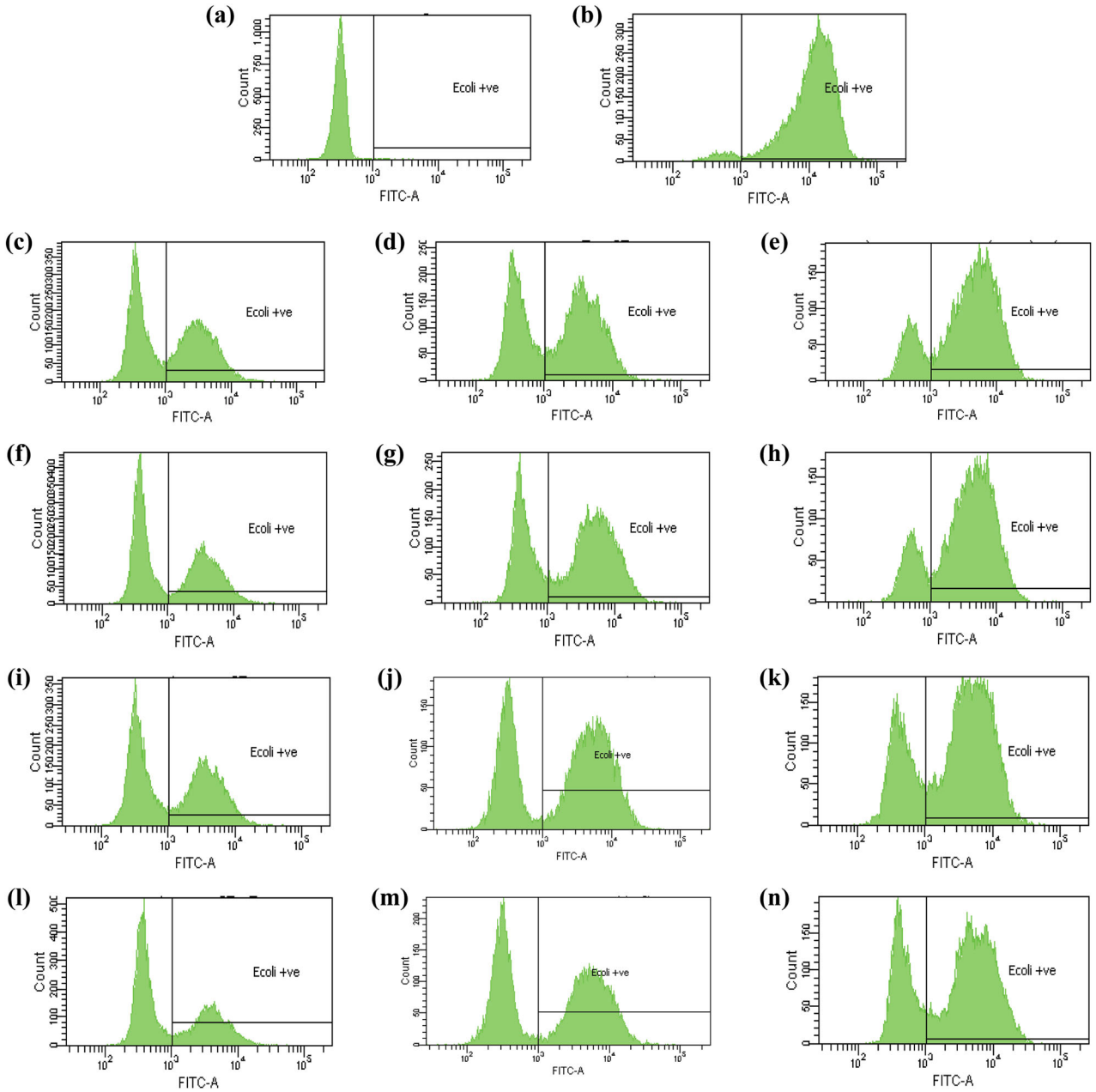

Fig. 4 Flow cytometer representation of *E. coli* engulfment by neutrophils. Inhibitory effects of α, β-unsaturated carbonyl-based compounds and pyrazoline derivatives at 100, 25, and 6.25 µg/mL on PMN phagocytic activity. (**a**) negative control, (**b**) positive control, (**c**) comp **4**(100 µg/mL), (**d**) comp **4**(25 µug/mL), (**e**) comp **4**(6.25 µg/mL), (**f**) comp **5**(100 µg/mL), (**g**) comp **5**(25 µg/mL), (**h**) comp **5**(6.25 µg/mL), (**i**) comp **8**(100 µg/mL), (**j**) comp **8**(25 µg/mL), (**k**) comp **8**(6.25 µg/mL), (**l**) comp **21**(100 µg/mL), (**m**) comp **21**(25 µg/mL), (**n**) comp **21** (6.25 µg/mL)

production. For the synthetic chalcones, the inhibitory effects of the compounds were less except for compounds **32** (15.8 µM) and **33** (16.73 µM) which showed comparable activity to aspirin i.e., 15.24 µM. for intracellular ROS production. However, all the synthetic chalcones exhibited strong extracellular ROS production in the whole blood with IC_{50} values ranging from 5.49 to 17.19 µM. Evident by previous studies, acetylsalicylic acid effectively inhibited oxidative burst stimulated by both zymosan and PMA.

The compounds were further and specifically investigated for their inhibitory effects on the oxidative burst by PMNs. All the compounds particularly compounds **4**, **11**, **13**, **22**, **25**, and **27** (IC_{50} values ranging from 3.12–4.77 µM) presented strong inhibition as curcumin (4.0 µM) in a concentration-dependent manner for luminol-enhanced chemiluminescence assay whereas for extracellular oxidative burst (lucigenin-induced), compounds **4, 14**, and **27** (5.26–6.10 µM) demonstrated high inhibitory activity,

Table 4 IC_{50} values (μM) of ROS inhibitory activity of α, β-unsaturated carbonyl-based compounds and pyrazoline derivatives on human whole blood and PMNs assayed by luminol and lucigenin amplified chemiluminescence

Compounds	Intracellular IC_{50} values (μM)		Extracellular IC_{50} values (μM)	
	Whole blood	PMNs	Whole blood	PMNs
1	–	47.93 ± 1.5	–	–
2	–	52.8 ± 1.2	–	–
3	–	7.19 ± 1.0	5.84 ± 0.5	12.2 ± 0.2
4	–	4.53 ± 0.8	14.4 ± 0.6	6.10 ± 0.1
5	3.13 ± 0.4	5.0 ± 0.2	55.8 ± 1.4	12.8 ± 0.3
6	–	14.5 ± 1.1	–	–
7	–	34.5 ± 1.3	–	–
8	–	9.17 ± 0.2	33.92 ± 0.6	33.68 ± 0.8
9	–	–	18.58 ± 0.2	37.24 ± 0.8
10	–	–	45.7 ± 0.9	–
11	3.97 ± 1.1	4.77 ± 0.2	12.36 ± 0.3	58.11 ± 1.1
12	–	–	–	–
13	4.02 ± 0.8	4.59 ± 0.6	11.56 ± 0.2	42.8 ± 0.6
14	3.84 ± 0.6	6.28 ± 0.1	20.42 ± 0.6	4.58 ± 1.1
15	–	9.06 ± 0.8	–	9.27 ± 1.1
16	6.37 ± 0.8	4.93 ± 0.2	–	70.09 ± 1.5
17	–	–	32.5 ± 0.7	36.22 ± 1.2
18	6.66 ± 0.4	10.88 ± 0.8	7.24 ± 0.3	12.15 ± 0.9
19	–	–	61.0 ± 1.5	–
20	25.3 ± 1.3	18.85 ± 0.8	–	22.05 ± 0.7
21	–	11.12 ± 0.2	31.14 ± 1.1	8.19 ± 0.5
22	7.33 ± 0.8	3.12 ± 0.1	60.17 ± 1.5	8.51 ± 0.3
23	–	18.34 ± 0.8	5.92 ± 0.8	25.0 ± 1.0
24	5.14 ± 1.0	14.82 ± 1.2	12.5 ± 1.0	16.27 ± 0.8
25		4.39 ± 0.4	12.34 ± 1.0	–
26			80.32 ± 1.1	
27	–	3.15 ± 0.9	–	5.26 ± 0.4
28	31.00 ± 0.4	43.20 ± 0.9	–	60.57 ± 1.2
29	–	6.08 ± 0.5	31.6 ± 1.0	25.74 ± 0.2
30	–	84.26 ± 1.2	–	–
31	–	–	14.6 ± 0.2	58.3 ± 1.4
32	15.80 ± 0.3	16.3 ± 0.6	15.5 ± 0.7	–
33	16.73 ± 0.4	11.67 ± 0.1	14.8 ± 0.4	11.73 ± 0.8
34	–	–	8.74 ± 0.1	4.51 ± 0.2
35	–	–	17.19 ± 0.8	18.6 ± 0.6
36	–	–	6.38 ± 0.2	41.5 ± 0.9
37	28.60 ± 0.2	–	7.54 ± 0.3	51 ± 1.4
38	–	–	15.16 ± 1.0	–
39	19.16 ± 0.1	–	5.98 ± 0.5	53 ± 1.2
40	–	–	15.9 ± 1.2	–
41	–	48.5 ± 1.1	8.91 ± 0.9	26.3 ± 0.8
Aspirin	15.24 ± 0.2	10.74 ± 0.4	43.6 ± 1.5	8.57 ± 0.3
Curcumin	7.4 ± 0.1	4.0 ± 0.1	14.1 ± 0.2	10.8 ± 0.1

(–) IC_{50} values were not determined

signifying that these compounds were more efficacious than the positive control, aspirin and curcumin which exhibited IC_{50} values of 8.5 and 10.8 μM, respectively, for lucigenin-amplified chemiluminescence. Among the chalcones analogs, compound **34** showed the strongest inhibition of ROS production with an IC_{50} value of 4.51 μM, stimulated by lucigenin. The inhibitory effects of compound **33** with IC_{50} values of 11.67 and 11.73 μM for intracellular and extracellular ROS production, respectively, was also comparable to aspirin and curcumin.

The professional phagocytic cells after being activated by serum-opsonized zymosan tend to accumulate at the site of infection and phagocytose the attacking microorganisms followed by the release of superoxide radicals and other secondarily derived ROS, such as H_2O_2, hypochlorous acid, and chloramines formed by NADPH oxidase enzyme. NADPH oxidase is present in both phagosome and plasma membrane and produces ROS both extracellularly and intracellularly. In addition, H_2O_2 diffuses freely through membranes and causes the release of ROS from the cell. Extracellularly released ROS could cause tissue damage and worsen inflammation while intracellular ROS kill phagocytosed pathogens or help the cell to kill them by chlorination of the protein content of neutrophil phagosome or by some other way. Therefore, it is important to differentiate between the intracellular and extracellular production of ROS (Rajecky et al. 2012). Owing to its small molecular weight, luminol was used as a probe to quantify intracellular ROS production. Therefore, the remarkable inhibition of the reactive species by the novel synthetic compounds could be attributed to their ability to block the interaction of zymosan with complement receptors, ultimately inhibiting NADPH oxidase (Rathakrishnan and Tiku 1993).

Structure activity analysis of α, β-unsaturated carbonyl-based compounds

α, β-Unsaturated carbonyl-based compounds possess remarkable reactivity and have been known for their diverse pharmacological activities conspicuously on immune system and are considered as valuable immunomodulators. Interestingly, these synthetic compounds exhibited variable activity trends for different steps of phagocytosis. All the compounds showed moderate to strong inhibition for the migration of PMNs, phagocytic activity and ROS production, nevertheless, very less activity was observed for CD11b/CD18 expression of leukocytes. In perspective of structure-activity relationship, compound **15**, with 1-benzyl-4-piperidone linker, showed good suppression on CD11b/CD18 integrin expression, though the remaining compounds did not display much activity.

The structural components found to be responsible for the increased chemotactic inhibition by the compounds

shown in Fig. 1 (compounds **1**–**30**) was N-methyl-4-piperidone linker and 4-piperidone moiety which was in accordance with our previous study. The strong suppressive activity seen by compound **6** can be attributed to the presence of diethoxymethyl group at position 4 on the benzene ring. Synthetic chalcones **31**, **32**, **37**, **38**, **39**, and **41** bear 2,3-dimethoxy substitution pattern on naphthalene ring and compounds **38**, **39**, and **37** also possess benzofuran ring attached to an aromatic ring. These structural features may be responsible for the inhibition of the chemotactic activity. Compound **41** bearing 2-pyrazoline-1-carboxamide as a linker, contains benzodioxane ring which has been reported to be an important feature for the anti-inflammatory activity of the synthesized compounds. Previously Bukhari et al. (2015a, 2015b) suggested that compounds belonging to 2-pyrazoline-1-carboxamide derivatives (**38**–**41**) and 2-pyrazoline-1-carbothioamide derivatives (**35**–**37**) were potent anti-inflammatory agents.

Compound **5** with the 1-benzyl-4-piperidone linker along with compound **4** (N-methyl-4-piperidone linker) and compound **8**, substituted with diethoxymethyl group at position 4 on the benzene ring have indicated potential phagocytic inhibitory effects. In addition, the results also showed that compound **21** was found highly active towards the inhibition of phagocytosis by PMNs. These results suggested that substitution of the phenyl ring at position 4 is important for good inhibitory activity. 2-Pyrazoline-1-carbothioamide derivatives and 2-pyrazoline-1-carboxamide derivatives also displayed good inhibition of phagocytosis by PMNs and have been documented as strong anti-inflammatory agents (Bukhari et al. 2015a, 2015b).

An insight into the structural features with respect to the potential activity revealed that presence of 2-nitro and 4 diethylamine substitution pattern (compound **11**) impart a role in the activity of compounds. Likewise, SAR studies of chemiluminescence assay outcomes also witnessed similar findings. Compound **14** having N-methyl-4-piperidone linker with substituent NCH_3 and compounds **3** and **23** having 4-piperidone linker with substituent NH were found most active in luminol- and lucigenin-enhanced chemiluminescence assay in both human whole blood and PMNs, respectively. Though compounds with the aforesaid linkers but with different substituents (**4**, **24**, and **13**) also showed higher inhibition than aspirin. Further analysis of results revealed that ROS production by PMNs stimulated by zymosan was also actively inhibited by compounds **11**, **22**, **25**, **27**, and **29**. Moreover, compounds **8, 9, 15, 16**, and **21** also exhibited good suppression of oxidative burst which may be due to the effect of substitution (phenyl-4-pyrrolidine) on aromatic rings of compounds. In the present study, the findings suggested that in addition to specific linkers imperative for activity, the substitution patterns on the linked aromatic rings also imparted more or less role in determining good activity.

Therefore, as an overall trend, N-methyl-4-piperidone, 4-piperidone and 1-benzyl-4-piperidone linkers remained important in showing highest inhibitory effects along with diethoxymethyl benzene on position 4 and phenyl 2-nitro 4-dimethylamine substitution pattern. For compounds **33** and **37** it was observed that substitution of methoxy group at position 2 and 3 of naphthalene ring showed strong anti-inflammatory properties compared to when the methoxy group was present at position 4 and 7. Further analysis of SAR indicated that 4-dimethylamino is also responsible for good inhibitory effects as shown by compound **34** and **38**.

Conclusion

Compounds with N-methyl 4-piperidone and 4-piperidone linkers (**4**, **13**, **14**, **23**,and **24**) revealed high inhibition on ROS production stimulated by both serum opsonized zymosan and PMA. In addition to N-methyl 4 piperidone linker and 4-piperidone linker, compounds with acetone linkers (**5**, **15**) and 2-pyrazoline-1-carboxamide derivatives (**2b, 4b**) also showed high inhibitory effects towards the chemotactic and phagocytic activity. The presence of diethoxymethyl-benzene at position 4 (**8**, **6**, **27**) and phenyl-2-nitro-4-dimethylamine (**11**, **13**, **14**, and **16**) were also found to be responsible for the immunosuppressive activity. Further mechanistic studies should be carried out to develop these compounds into potent immunosuppressive agents in treating different immune-related diseases particularly related to inflammatory disorders.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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