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Original article

Synthetic 2′,5′-dimethoxychalcones as G_2/M arrest-mediated apoptosis-inducing agents and inhibitors of nitric oxide production in rat macrophages

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Abstract

In an effort to develop novel antitumor or chemopreventive agents, a series of 2′,5′-dimethoxychalcone derivatives were prepared by Claisen–Schmidt condensation of appropriate acetophenones with suitable aromatic aldehyde. In vitro screening revealed low micromolar activity (IC_{50}) against several human cancer lines. Activity in MCF-7 cells correlated with the ability to induce G_2/M arrest-mediated apoptosis following drug treatment by the most potent agent, **8**, an observation further reinforced by fluorescence microscopy. Compounds **3**, **8**, and **10** showed potent inhibitory effect on NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage-like cells. The present results demonstrated that **3**, **8**, and **10** are potential anti-inflammatory and cancer chemopreventive agents.
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Keywords: 2′,5′-Dimethoxychalcones; G_2/M arrest; Apoptosis; NO; Anti-inflammatory effect

1. Introduction

Chalcones have displayed a variety of biological activities, among which anti-malaria, anti-protozoal, anti-inflammatory, immunomodulatory, nitric oxide inhibition, tyrosinase inhibition, cytotoxic, anticancer, and antileishmanial activities have been cited in the literature [1–3]. A series of synthetic chalcones were reported as antimitotic agents [4]. One of these, (*E*)-1-(2,5-dimethoxyphenyl)-3-[4-(dimethylamino)-phenyl]-2-methyl-2-propen-1-one) (**1**), was reported to be an effective antimitotic agent at a concentration of 4 μM in an in vitro Hela cell test system [4]. Our recent report has demonstrated that some synthetic chalcone derivatives showed selective cytotoxicity against MCF-7 cells [4]. One of the chalcone, 2′,5′-dimethoxy-4-hydroxychalcone (**2**), showed potent inhibitory effect on NO accumulation from RAW 264.7 cells, and manifested potent selective cytotoxicity against human MCF-7 cells and caused cell death by apoptosis [5]. These findings suggested that some chalcones might be promising antitumor or cancer chemopreventive agents and have potential in the therapy of tumor. This report described the chemistry of further synthesized series of 2′,5′-dimethoxychalcone derivatives, cytotoxic activity, anti-inflammatory activity and the structure–cytotoxic or anti-inflammatory activity relationships of these series of novel antitumor or cancer chemopreventive agents.

2. Chemistry

As depicted in Scheme 1 for the specific synthesis of new chalcone **8**, we have prepared a number of new 2′,5′-dimethoxychalcones (**3–10**) using Claisen–Schmidt condensation of

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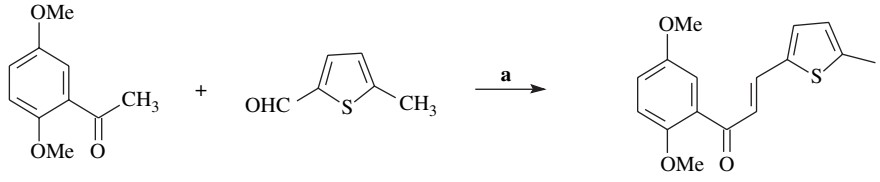


8a **8b** **8**

Scheme 1. (a) Reagents and condition: BaOH, $8H_2O$, 40 °C, HCl.

2′,5′-dimethoxyacetophenone (**8a**) with appropriate aromatic aldehyde (**8b**) [5]. For the synthesis of new chalcones **12**–**14** using compound **2** reacted with appropriate cinnamoyl chloride. New chalcone **11** was prepared as depicted in Scheme 2. These reactions afforded various 2′,5′-dimethoxychalcones in a good yield except for compounds **11** and **14** (Table 1).

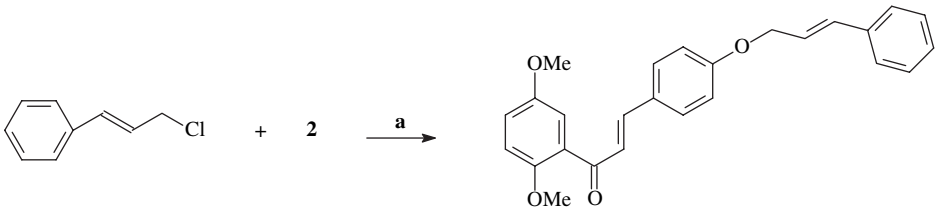
3. Biological results and discussion

Evaluation of the growth-inhibitory properties of 2′,5′-dimethoxychalcones was undertaken in four human cancer cell lines, A549 (lung), Hep 3B (liver), HT-29 (colorectal), and MCF-7 (breast), using a MTT microassay for cytotoxicity and following the method described previously [6]. The results are listed in Table 2. A number of compounds indicated significant inhibitory activities against several cancer cell types. All compounds in Table 2 showed selective cytotoxicity against MCF-7 cells except for compounds **6** and **8** which showed significant activity against all the cell lines used in Table 2. Compounds with a thienyl moiety revealed significant cytotoxic activity and showed stronger cytotoxic activity than those of compounds with a furfuryl moiety except for compound **9**. Compound with a 3-thienyl moiety, such as **6**, indicated stronger cytotoxic activity than that of compound with a 2-thienyl moiety such as **5**. A methyl group substituted at C-5 of compound with a 2-thienyl moiety, such as **8**, significantly enhanced the cytotoxicity while a methyl group substituted at C-3 of compound with same skeleton, such as **9**, did not enhance the cytotoxicity. The B ring of **2** replaced with 3-thieny moiety or 2-(5-methylthienyl) moiety, such as **6** or **8**, significantly enhanced the cytotoxicity against Hep 3B and HT-29 cell lines while it did not enhance the cytotoxicity against MCF-7 cells. The etherification or esterification of phenolic group at C-4 of **2** by cinnamyl chloride or cinnamoyl chloride did not enhance the cytotoxicity against the cell lines used in Table 2.

It has been recognized that apoptotic cells reduced DNA stainability with a variety of fluorochromes [7]. The appearance of cells with low DNA stainability forms a ''sub-G_1 peak'', which has been considered to be the hallmark of all death by apoptosis [8]. In this study, various concentrations of **8** were added to MCF-7 cells for different time periods. As shown in Fig. 1, a sub-G_1 peak was detected in the DNA histograms of **8** at various concentrations for different time periods. The shift of G_0/G_1 and S phase cell cycles to the G_2/M phase is decreased dose dependently in the MCF-7 cell treated by **8** for different time periods, while a significant G_2/M accumulation was observed at 24 h and 48 h time periods, respectively, following the administration of **8**. However, a maximum of $63.79 \pm 5.1\%$ apoptosis cells were detected at 72 h. It seems that treatment with **8** was associated with accumulation of cells at G_2/M which in part appeared to be due to mitotic arrest prior to metaphase.

Within various time periods, Hoechst 33258 staining of cells exposed to various concentrations of **8** demonstrated typical morphological changes of apoptosis (Fig. 2). The cell shrunk, turned round, and had a relatively smaller volume than control cells. Chromatin DNA was extensively condensed and DNA was fragmented. The typical morphological and biochemical features clearly revealed that **8** causes cell death by apoptosis.

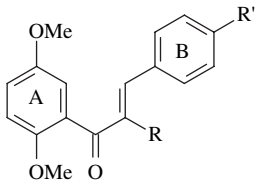
Previously we report compound **2** revealed the shift of G_0/G_1 and G_2/M cell cycles to the sub-G_1 phase and caused cell death by apoptosis [5]. The B ring of **2** replaced by a 2-(5-methyl)-thienyl moiety, such as **8**, may induce G_2/M phase arrest and caused cell death by apoptosis. Further experiments are needed to elucidate the mechanism of action of **8**. The anti-inflammatory activities of randomly selected compounds **3**, **5**, **7**, **8**, **10**,



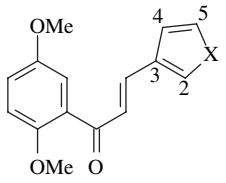
11a **11**

Scheme 2. (a) Reagents and condition: KOH, MeOH, reflux 1 h.

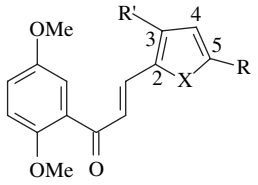
Table 1
Structure and analytical data of 2′,5′-dimethoxychalcones



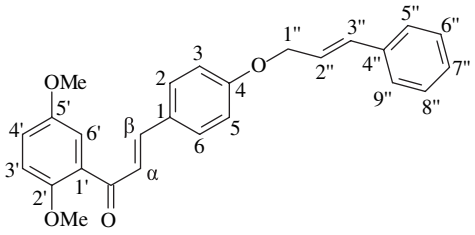
1 (R = CH_3, R' = $N(CH_3)_2$)
2 (R = H, R' = OH)



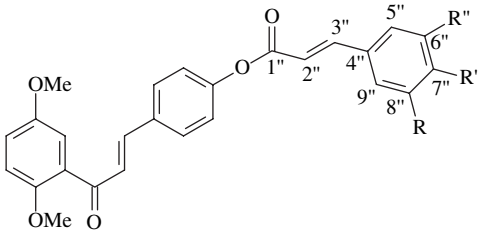
4,6



3,5,7-10



11



12-14

Compound	R	R′	R″	X	Formula	Mp °C	Yield %	Analysis[a]
3	H	H		O	$C_{15}H_{14}O_4$	Oil	41	C, H
4				O	$C_{15}H_{14}O_4$	Oil	33	C, H
5	H	H		S	$C_{15}H_{14}O_3S$	70–72	50[b]	C, H, S
6				S	$C_{15}H_{14}O_3S$	Oil	42	C, H, S
7	Me	H		O	$C_{16}H_{16}O_4$	64–65	58[b]	C, H
8	Me	H		S	$C_{16}H_{16}O_3S$	74–75	56[b]	C, H, S
9	H	Me		S	$C_{16}H_{16}O_3S$	Oil	31	C, H, S
10	Br	H		O	$C_{15}H_{13}BrO_4$	Powder	30	C, H
11					$C_{26}H_{24}O_4$	118–120	23[b]	C, H
12	H	Me	H		$C_{27}H_{24}O_5$	116–119	41[b]	C, H
13	H	OMe	H		$C_{27}H_{24}O_6$	140–143	40[b]	C, H
14	OMe	OMe	OMe		$C_{29}H_{28}O_8$	153–155	11[b]	C, H

[a] C, H and S analyses were within ±0.4% of the theoretical values.
[b] Solvents used for recrystallization: $CHCl_3$.

Table 2
Cytotoxicity of **3**–**14** (IC_{50} values in μM)[a]

Compound	Cell line			
	A549	Hep 3B	HT-29	MCF-7
3	27.9 ± 1.5	19.0 ± 1.2	24.8 ± 1.4	10.5 ± 0.9
4	22.1 ± 0.9	12.0 ± 0.7	21.3 ± 1.5	18.2 ± 0.7
5	11.0 ± 1.1	8.4 ± 0.9	13.3 ± 1.3	3.8 ± 0.08
6	3.8 ± 0.3	5.7 ± 0.6	5.3 ± 0.2	3.7 ± 0.1
7	51.5 ± 2.1	26.5 ± 1.2	43.0 ± 2.3	10.3 ± 0.4
8	4.5 ± 0.09	3.4 ± 0.01	1.8 ± 0.03	2.6 ± 0.08
9	22.2 ± 1.1	16.7 ± 0.7	19.4 ± 1.4	7.6 ± 0.3
10	14.9 ± 1.3	11.0 ± 1.7	15.2 ± 2.1	11.0 ± 0.9
11	39.8 ± 2.7	17.3 ± 1.9	26.3 ± 2.4	19.0 ± 1.5
12	29.7 ± 2.2	29.0 ± 2.5	16.4 ± 1.5	12.6 ± 1.1
13	60.4 ± 4.1	42.6 ± 3.8	23.0 ± 2.0	4.1 ± 0.1
14	36.7 ± 3.6	23.2 ± 2.1	23.8 ± 2.5	6.4 ± 0.5
5-Fu	3.1 ± 0.2	0.6 ± 0.3	0.6 ± 0.1	1.5 ± 0.1

[a] Data are presented as mean ± SD ($n = 3$). Compounds **3**–**14** or 5-Fu dissolved in DMSO, were diluted with culture medium containing 0.1% DMSO respectively. The control cells were treated with culture medium containing 0.1% DMSO. 5-Fu (5-fluorouracil) was used as a positive control.

and **11** were studied in vitro for their inhibitory effects on chemical mediators released from mast cells, neutrophils and macrophages. Compound **3** had significant and concentration-dependent inhibitory effect on the release of β-glucuronidase from rat mast cell stimulated with compound 48/80 (Table 3). It indicated that the B ring of 2′,5′-dimethoxychalcones replaced by an unsubstituted 2-furan ring such as **3** revealed significant inhibition of mast cell degradation. Formyl-Met-Leu-Phe (FMLP) (1 μM)/cytochalasin B (CB) (5 μg ml^{-1}) stimulated the release of β-glucuronidase and lysozyme from rat neutrophils. Compounds **3** and **7** had potent and concentration-dependent inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP/CB, respectively (Table 4), while compound **5** showed significant inhibitory effect on the release of lysozyme from rat neutrophils stimulated with fMLP/CB. Based on the data appeared on the Table 4, it clearly indicates that the 2′,5′-dimethoxychalcones with an unsubstituted or a 5-methyl-2-furan ring enhances the inhibition of rat neutrophil degranulation.

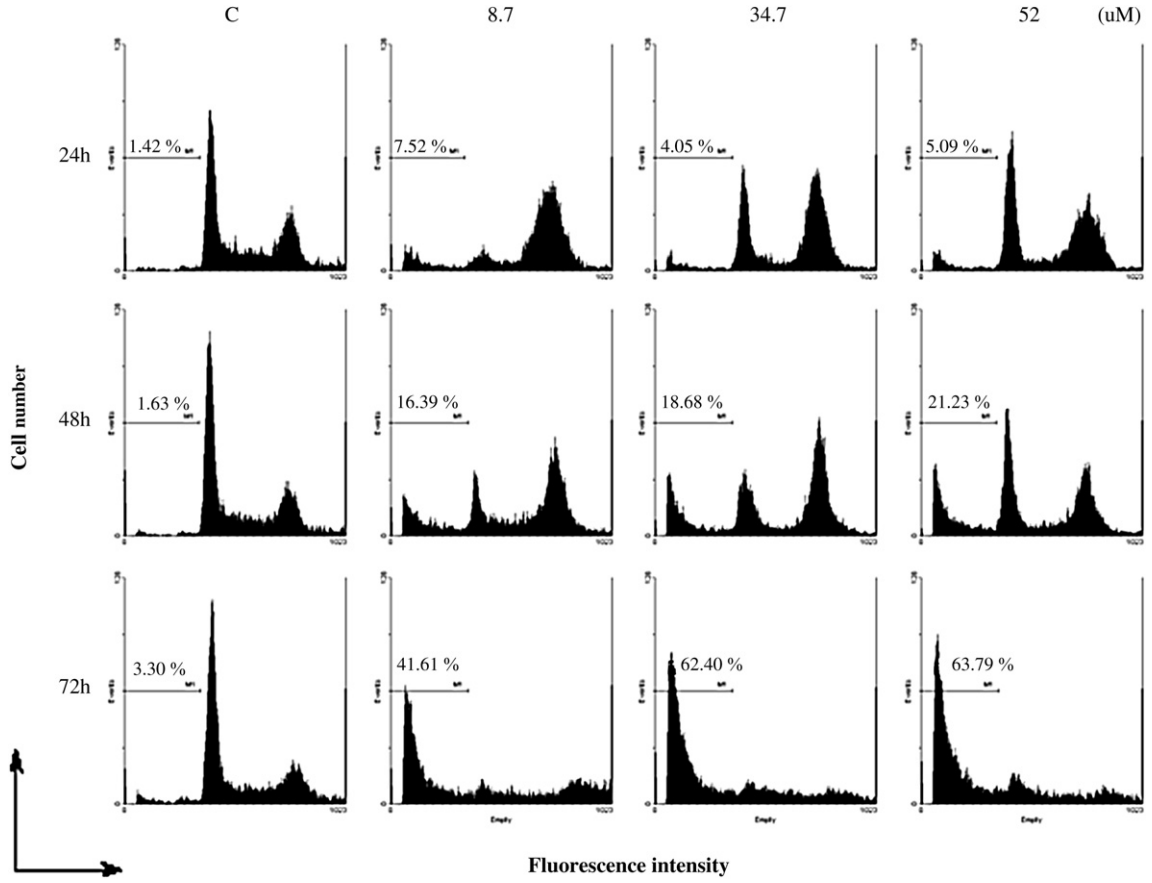


Fig. 1. Flow cytometry analysis of **8**-treated MCF-7 cells. MCF-7 (1×10^4 cells/mL) was treated with various concentrations of **8** for different time periods. At the times indicated, cells were stained with propidium iodide (PI), and DNA contents were analyzed by flow cytometry, apoptosis was measured by the accumulation of sub-G_1 DNA contents in cells. The control cells were treated with medium. Results were representative of three independent experiments.

FMLP (0.3 μM)/CB (5 μg ml^{-1}) or phorbol myristate (PMA; 3 nM) stimulated superoxide anion generation in rat neutrophils. As shown in Table 5, compounds **3** and **10** had significant and concentration-dependent inhibitory effects on fMLP/CB-induced superoxide anion generation. Based on the data shown on Table 5, it indicates that 2′,5′-dimethoxychalcones with an unsubstituted or a 5-methyl-2-furan ring enhances the inhibitory effect on fMLP/CB-induced superoxide anion generation in rat neutrophils while it does not enhance the inhibitory effect on PMA-induced superoxide anion generation.

As shown in Table 5, compounds **3**, **5**, **7**, **8**, **10**, and **11** showed different inhibitory effects on superoxide anion formation from rat neutrophils caused by fMLP/CB and PMA. It further established that fMLP and PMA elicit the superoxide anion formation from neutrophils through a different transduction mechanism and are regulated differently [9].

Treatment of RAW 264.7 macrophage-like cells with lipopolysaccharide (LPS; 1 μg ml^{-1}) for 24 h induced NO production as assessed by measuring the accumulation of nitrite, a stable metabolite of NO in the media, based on Griess [10]. As shown in Table 6, compounds **3**, **8**, and **10** showed potent and concentration-dependent inhibitory effects on NO accumulation from RAW 264.7 cells. NO plays a central role in macrophage-induced cytotoxicity and expressed NO may contribute to the pathophysiology of septic shock [11]. The excessive production of NO also can destroy functional normal tissue during acute and chronic inflammation [12]. The inhibition of NO production by **8** in macrophages and significant cytotoxic activity by **8** against the cell lines listed in Table 2 suggest that **8** may be used as cancer chemopreventive drugs.

Effect on the generation of tumor necrosis factor-α (TNF-α) was determined in RAW 264.7 cells activated by LPS [13–15]. As shown in Table 6, compound **3** strongly inhibited TNF-α generation in LPS-stimulated RAW 264.7 cells. Further experiments are needed to elucidate the mechanism of actions of cytotoxic and anti-inflammatory effects of these compounds.

4. Conclusions

In this study we have synthesized a novel family of 2′,5′-dimethoxychalcones and studied their ability to inhibit the growth in human tumor cell lines. This series of compounds revealed potent and selective cytotoxicity against human MCF-7 cells. The most potent compound **8** showed G_2/M arrest-mediated apoptosis in human MCF-7 cell lines. This compound has the potential to develop as a new class of antimitotic agent. The present study also suggested that the inhibition of

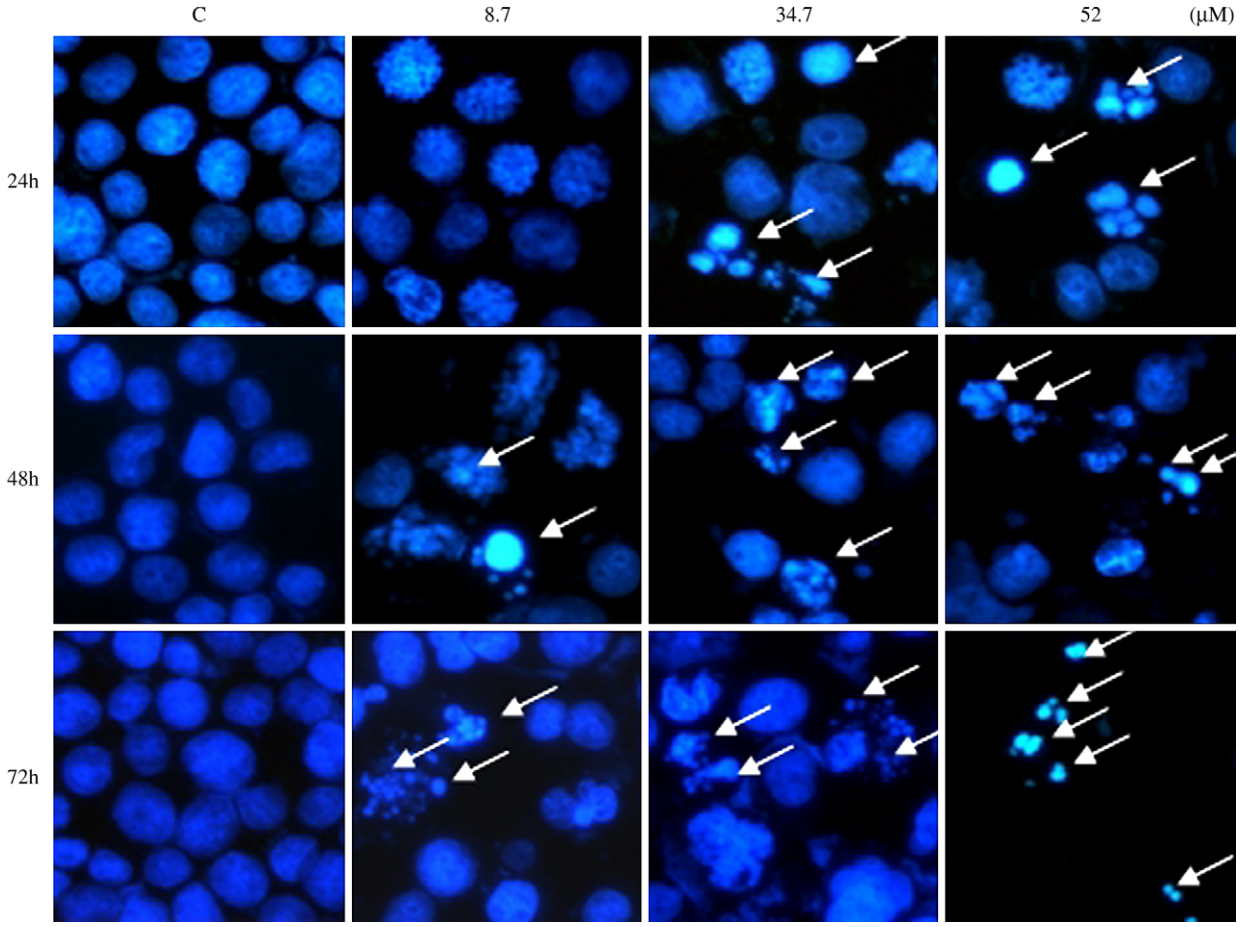


Fig. 2. Fluorescence microscopy observation of chromatin condensation in MCF-7 cells induced by **8**. Co-staining of Hoechst 33258 and PI showed shrunken nuclei and nuclear fragmentation in MCF-7 cells treated with **8** (8.7, 34.7, and 52 μM). The control cells (C) were treated with the medium.

NO production by **3**, **8**, and **10** in macrophages may be used as cancer chemopreventive drugs.

5. Experimental protocols

Melting points (uncorrected) were determined with a Yanco micro melting point apparatus. Refractive indexes were determined with an ABBE refractomer, Model 1T, ATAGO. IR spectra were determined with a Perkin–Elmer system 2000 FTIR spectrophotometer. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian UNITY-400 spectrometer, and mass spectra were obtained on a JMX-HX 100 mass spectrometer. Elemental analyses were within ±0.4% of the theoretical values, unless otherwise noted.

Table 3
The inhibitory effects of dimethoxychalcones on the release of β-glucuronidase and histamine from rat mast cells stimulated with compound 48/80 (10 μg ml^{-1})

Compound	IC_{50} (μM)[a]	
	β-Glucuronidase	Histamine
3	18.4 ± 5.4	>30 (38.5 ± 6.6)
5	>30 (−4.6 ± 12.4)	>30 (0.8 ± 2.5)
7	>30 (12.7 ± 12.0)	>30 (17.5 ± 12.4)
8	>30 (−2.7 ± 8.8)	>30 (43.5 ± 1.3)
10	>30 (−13.3 ± 7.9)	>30 (6.3 ± 3.9)
11	>30 (−11.2 ± 12.5)	>30 (0.3 ± 3.3)
Acetylshikonin	2.2 ± 0.7	2.0 ± 0.4

Data are presented as mean ± SD ($n = 3$). Acetylshikonin was used as a positive control.
[a] When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses.

Table 4
The inhibitory effects of dimethoxychalcones on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP (1 μM)/CB (5 μg ml^{-1})

Compound	IC_{50} (μM)[a]	
	β-Glucuronidase	Lysozyme
3	6.2 ± 0.8	8.3 ± 4.4
5	>30 (38.5 ± 9.1)	14.6 ± 13.1
7	22.2 ± 5.1	17.8 ± 7.2
8	>30 (45.6 ± 4.7)	>30 (37.4 ± 5.4)
10	>30 (36.9 ± 12.2)	>30 (33.4 ± 15.7)
11	>30 (18.8 ± 7.6)	>30 (33.1 ± 13.1)
TFP	13.2 ± 1.2	12.7 ± 3.5

Data are presented as mean ± SD ($n = 3$). Trifluoperazine (TFP) was used as a positive control.
[a] When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses.

Table 5
The inhibitory effects of dimethoxychalcones on superoxide anion generation (nmol) in rat neutrophils stimulated with fMLP (0.3 μM)/CB (5 μg ml^{-1}) or PMA (3 nM)

Compound	IC_{50} (μM)[a]	
	FMLP	PMA
3	3.0 ± 1.8	>30 (49.1 ± 4.1)
5	>30 (47.2 ± 8.2)	>30 (7.9 ± 5.3)
7	>30 (48.1 ± 1.1)	>30 (39.0 ± 13.7)
8	>30 (49.1 ± 8.6)	>30 (−11.9 ± 10.2)
10	19.7 ± 4.6	>30 (−2.2 ± 14.7)
11	>10 (−71.9 ± 15.1)	>10 (4.1 ± 2.9)
DPI	5.8 ± 2.8	3.3 ± 0.9

Data are presented as mean ± SD ($n = 3$). Diphenylene iodonium chloride (DPI) was used as a positive control.
[a] When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses.

Chromatography was performed using a flash-column technique on silica gel 60 supplied by E. Merck.

5.1. General procedure for obtaining chalcones **3–14**

Appropriate aldehyde (25 mmol), barium hydroxide octahydrate (25 mmol) and 2′,5′-dimethoxyacetophenone (25 mmol) were dissolved in MeOH (100 ml). The mixture was stirred for 12 h at 40 °C and then evaporated in vacuo, water (100 ml) was added and the mixture was neutralized with HCl (1 M, 35 ml) and extracted with EtOAc. The organic layer was separated, washed with water, dried, and evaporated in vacuo. The residue was chromatographed over a silica gel column and eluted with appropriate solvent to yield compounds **3–10**. Compound **2** (25 mmol) was dissolved in MeOH (100 ml) and KOH (25 mmol) and cinnamyl chloride (25 mmol) were added and refluxed for 1 h. The mixture was evaporated in vacuo, neutralized with 1 M HCl, and extracted with EtOAc. The organic layer was separated and evaporated in vacuo. The residue was chromatographed over a silica gel column and eluted with appropriate solvent to yield **11**. Various substituted cinnamic acid (3.2 g, 25 mmol) in anhydrous C_6H_6 (60 ml) was treated with 5.0 ml of oxalyl chloride under an Ar atmosphere and thoroughly stirred at room temperature. After 2 h of stirring, the solvent and the excess reagent were removed under reduced pressure. The residue was dissolved in anhydrous pyridine (100 ml) and reacted with **2** (25 mmol). The mixture was refluxed for 1 h and then evaporated in vacuo. The residue was chromatographed over a silica gel column and eluted with appropriate solvent to yielded **12–14**.

Table 6
The inhibitory effects of dimethoxychalcones on the accumulation of NO_2^- and TNF-α formation in the culture media of RAW 264.7 cells in response to LPS (1 μg ml^{-1})

Compound	IC_{50} (μM)[a]	
	NO_2^-	TNF-α
3	5.7 ± 2.0	4.6 ± 0.1
5	>10 (49.8 ± 9.0)	>30 (5.9 ± 2.0)
7	>10 (29.5 ± 3.5)	>10 (−10.5 ± 4.2)
8	6.7 ± 1.2	>30 (46.0 ± 15.0)
10	12.8 ± 4.7	>30 (35.6 ± 12.2)
11	>30 (27.9 ± 4.2)	>30 (15.0 ± 13.1)
Positive control	5.2 ± 1.1	18.1 ± 2.5

Data are presented as mean ± SD ($n = 3$). *N*-(3-Aminomethyl) benzylacetamidine (1400w) and genistein were used as a positive control for the accumulation of NO_2^- and TNF-α formation in the culture media of RAW 264.7 cells in response to LPS, respectively.
[a] When 50% inhibition could not be reached at the highest concentration, the percentage of inhibition is given in parentheses.

5.1.1. 2′,5′-Dimethoxy-2-furfurylchalcone (**3**)

Furan-2-carbaldehyde (2.4 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **3** as yellow oil (2.64 g, 10.25 mmol, 41%), n_D^{27} 1.356. IR (KBr) 1654, 1593, 1493, 1280, 1223, 1035, 1017 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.78 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 6.58 (1H, dd, $J = 3.2$, 2.0 Hz, H-4), 6.88 (1H, d, $J = 3.2$, 0.9 Hz, H-3), 7.04 (1H, d, $J = 9.2$ Hz, H-3′), 7.06 (1H, dd, $J = 9.2$, 2.0 Hz, H-4′), 7.16 (1H, dd, $J = 2.0$, 0.9 Hz, H-5), 7.33 (1H, d, $J = 15.2$ Hz, H-α), 7.45 (1H, d, $J = 15.2$ Hz, H-β), 7.70 (1H, d, $J = 2.0$ Hz, H-6′). ^{13}C NMR ($CDCl_3$): δ 56.6 (OCH_3), 57.3 (OCH_3), 114.1 (C-6′), 114.9 (C-3′), 115.9 (C-3), 117.3 (C-4), 119.9 (C-α), 125.7 (C-4′), 130.2 (C-5), 131.1 (C-1′), 146.7 (C-β), 153.1 (C-2), 153.9 (C-5′), 155.1 (C-2′), 192.1 (C=O); MS (EI) *m/z* 258 (M^+, 100). Anal Calcd for $C_{15}H_{14}O_4$: C, 69.80; H, 5.50. Found: C, 69.35; H, 5.50.

5.1.2. 2′,5′-Dimethoxy-3-furfurylchalcone (**4**)

Furan-3-carbaldehyde (2.4 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **4** as yellow oil (2.13 g, 8.25 mmol, 33%), n_D^{26} 1.335. IR (KBr) 1665, 1594, 1493, 1227, 1223 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.70 (3H, s, OCH_3), 3.74 (3H, s, OCH_3), 6.57 (1H, m, H-4), 6.83 (1H, d, $J = 8.8$ Hz, H-3′), 6.90 (1H, dd, $J = 9.2$, 3.2 Hz, H-4′), 7.05 (1H, d, $J = 15.6$ Hz, H-α), 7.08 (1H, d, $J = 3.2$ Hz, H-5), 7.34 (1H, m, H-2), 7.46 (1H, d, $J = 15.6$ Hz, H-β), 7.61 (1H, s, H-6′). ^{13}C NMR ($CDCl_3$): δ 55.4 (OCH_3), 56.0 (OCH_3), 107.3 (C-4), 113.0 (C-6′), 114.1 (C-3′), 118.4 (C-α), 123.0 (C-3), 126.6 (C-4′), 129.2 (C-1′), 133.1 (C-2), 144.1 (C-β), 144.9 (C-5), 152.1 (C-5′), 153.2 (C-2′), 191.9 (C=O); MS (EI) *m/z* 258 (M^+, 100). Anal Calcd for $C_{15}H_{14}O_4$: C, 69.80; H, 5.50. Found: C, 69.55; H, 5.58.

5.1.3. 2′,5′-Dimethoxy-2-thienylchalcone (**5**)

2-Thiophenecarboxaldehyde (2.8 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **5** as yellow needles (3.42 g, 12.5 mmol, 50%). IR (KBr) 1650, 1581, 1493, 1413, 1280, 1221, 1035 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.78 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 6.92 (1H, d, $J = 8.8$ Hz, H-3′), 6.99 (1H, dd, $J = 8.8$, 3.2 Hz, H-4′), 7.03 (1H, dd, $J = 4.8$, 3.6 Hz, H-4), 7.18 (1H, d, $J = 3.2$ Hz, H-6′), 7.24

(1H, d, $J = 15.6$ Hz, H-α), 7.29 (1H, d, $J = 3.0$, 0.8 Hz, H-3), 7.37 (1H, d, $J = 4.8$ Hz, H-5), 7.76 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 55.7 (OCH_3), 56.3 (OCH_3), 113.3 (C-6′), 114.3 (C-3′), 119.1 (C-α), 125.8 (C-4′), 128.2 (C-3), 128.4 (C-4), 129.3 (C-1′), 131.4 (C-5), 135.5 (C-β), 140.5 (C-2), 152.5 (C-5′), 153.5 (C-2′), 191.6 (C═O); MS (EI) *m/z* 274 (M^+, 42). Anal Calcd for $C_{15}H_{14}O_3S$: C, 65.70; H, 5.10; S, 11.70. Found: C, 65.70; H, 5.02; S, 11.67.

*5.1.4. 2′,5′-Dimethoxy-3-thienylchalcone (**6**)*

3-Thiophenecarboxaldehyde (2.8 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **6** as yellow oil (2.88 g, 10.5 mmol, 42%), n_D^{26} 1.341. IR (KBr) 1653, 1586, 1493, 1282, 1222, 1035 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.80 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 6.93 (1H, d, $J = 8.8$ Hz, H-3′), 6.94 (1H, dd, $J = 8.8$, 2.8 Hz, H-4′), 7.16 (1H, d, $J = 2.8$ Hz, H-6′), 7.22 (1H, d, $J = 15.6$ Hz, H-α), 7.32 (1H, ddd, $J = 5.2$, 2.8, 0.4 Hz, H-4), 7.37 (1H, dd, $J = 5.2$, 1.6 Hz, H-5), 7.53 (1H, dd, $J = 2.8$, 1.6 Hz, H-2), 7.62 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 55.8 (OCH_3), 56.4 (OCH_3), 113.3 (C-6′), 114.3 (C-3′), 118.9 (C-α), 125.3 (C-5), 126.7 (C-4′), 126.8 (C-2), 128.7 (C-4), 129.6 (C-1′), 136.9 (C-β), 138.3 (C-2), 152.4 (C-5′), 153.5 (C-2′), 192.7 (C═O); MS (EI) *m/z* 274 (M^+, 59). Anal Calcd for $C_{15}H_{14}O_3S$: C, 65.70; H, 5.10; S, 11.70. Found: C, 65.26; H, 5.06; S, 12.10.

*5.1.5. 2′,5′-Dimethoxy-2-(5-methylfurfuryl)chalcone (**7**)*

5-Methyl-2-furaldehyde (2.75 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **7** as yellow needles (3.94 g, 14.5 mmol, 58%). IR (KBr) 1653, 1599, 1568, 1493, 1276, 1223, 1037, 1021 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.30 (3H, s, CH_3), 3.74 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 6.04 (1H, dd, $J = 3.2$, 0.8 Hz, H-3), 6.53 (1H, d, $J = 3.2$ Hz, H-4), 6.86 (1H, d, $J = 9.2$ Hz, H-3′), 6.93 (1H, dd, $J = 8.8$, 2.8 Hz, H-4′), 7.12 (1H, d, $J = 2.8$ Hz, H-6′), 7.16 (1H, d, $J = 15.6$ Hz, H-α), 7.32 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 13.9 (CH_3), 55.5 (OCH_3), 56.2 (OCH_3), 109.1 (C-α), 113.1 (C-6′), 114.2 (C-3′), 117.5 (C-3), 118.4 (C-4), 122.4 (C-4′), 129.6 (C-1′), 129.7 (C-β), 150.1 (C-5), 152.2 (C-2), 153.3 (C-5′), 155.5 (C-2′), 191.8 (C═O); MS (EI) *m/z* 272 (M^+, 100). Anal Calcd for $C_{16}H_{14}O_4$: C, 70.60; H, 5.90. Found: C, 70.50; H, 5.91.

*5.1.6. 2′,5′-Dimethoxy-2-(5-methylthienyl)chalcone (**8**)*

5-Methyl-2-thiophenecarboxaldehyde (3.15 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **8** as yellow needles (4.03 g, 14 mmol, 56%). IR (KBr) 1648, 1578, 1493, 1280, 1221, 1035 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.44 (3H, s, CH_3), 3.74 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 6.67 (1H, dd, $J = 3.6$, 1.2 Hz, H-3), 6.87 (1H, d, $J = 8.8$ Hz, H-3′), 6.95 (1H, dd, $J = 8.8$, 3.2 Hz, H-4′), 7.05 (1H, d, $J = 3.6$ Hz, H-4), 7.08 (1H, d, $J = 15.6$ Hz, H-α), 7.15 (1H, d, $J = 3.2$ Hz, H-6′), 7.65 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 15.6 (CH_3), 55.5 (OCH_3), 56.1 (OCH_3), 113.1 (C-6′), 114.2 (C-3′), 118.6 (C-α), 124.4 (C-3), 126.6 (C-4′), 129.3 (C-1′), 132.2 (C-4), 136.0 (C-β), 138.4 (C-2), 144.0 (C-5), 152.2 (C-5′), 153.3 (C-2′), 191.4 (C═O); MS (EI) *m/z* 288 (M^+, 28). Anal Calcd for $C_{16}H_{16}O_3S$: C, 66.60; H, 5.60; S, 11.10. Found: C, 66.66; H, 5.47; S, 10.92.

*5.1.7. 2′,5′-Dimethoxy-2-(3-methylthienyl)chalcone (**9**)*

3-Methyl-2-thiophenecarboxaldehyde (3.15 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **9** as yellow oil (2.23 g, 7.75 mmol, 31%), n_D^{26} 1.360. IR (KBr) 1648, 1575, 1493, 1281, 1222, 1038 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.35 (3H, s, CH_3), 3.80 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 6.87 (1H, d, $J = 4.8$ Hz, H-4), 6.92 (1H, d, $J = 8.8$ Hz, H-3′), 6.99 (1H, dd, $J = 9.2$, 3.2 Hz, H-4′), 7.20 (1H, d, $J = 4.8$ Hz, H-5′), 7.21 (1H, d, $J = 3.2$ Hz, H-6′), 7.25 (1H, d, $J = 15.6$ Hz, H-α), 7.86 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 14.2 (CH_3), 55.8 (OCH_3), 56.4 (OCH_3), 113.3 (C-6′), 114.3 (C-3′), 119.1 (C-α), 124.9 (C-4′), 127.0 (C-5), 129.6 (C-1′), 131.3 (C-4), 133.8 (C-β), 142.1 (C-2), 152.6 (C-5′), 153.5 (C-2′), 191.4 (C═O); MS (EI) *m/z* 288 (M^+, 59). Anal Calcd for $C_{16}H_{16}O_3S$: C, 66.60; H, 5.60; S, 11.10. Found: C, 66.30; H, 5.56; S, 11.02.

*5.1.8. 2′,5′-Dimethoxy-2-(5-bromofurfuryl)chalcone (**10**)*

5-Bromo-2-furaldehyde (4.37 g, 25 mmol), 2′,5′-dimethoxyacetophenone (4.5 g, 25 mmol) and barium hydroxide octahydrate (7.89 g, 25 mmol) were treated as in general method for the synthesis of chalcone to give **10** as brown powder (2.04 g, 7.5 mmol, 30%). IR (KBr) 1655, 1594, 1493, 1279, 1223 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.80 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.41 (1H, d, $J = 3.6$ Hz, H-4), 6.60 (1H, d, $J = 3.6$ Hz, H-3), 6.92 (1H, d, $J = 9.2$ Hz, H-3′), 7.00 (1H, dd, $J = 8.8$, 3.2 Hz, H-4′), 7.18 (1H, d, $J = 3.2$ Hz, H-6′), 7.28 (1H, d, $J = 15.6$ Hz, H-α), 7.33 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 55.8 (OCH_3), 56.4 (OCH_3), 113.3 (C-3′), 114.3 (C-6′), 114.4 (C-4), 117.5 (C-3), 119.4 (C-4′), 124.6 (C-α), 125.4 (C-2), 127.9 (C-β), 129.4 (C-1′), 152.7 (C-2′), 153.5 (C-5′), 153.7 (C-5), 191.4 (C═O); MS (EI) *m/z* 336 (M^+, 3). Anal Calcd for $C_{15}H_{13}BrO_4$: C, 53.40; H, 3.90. Found: C, 53.66; H, 3.94. The structure was supported by 2D spectra.

*5.1.9. 2′,5′-Dimethoxy-4-cinnamyloxychalcone (**11**)*

Cinnamyl chloride (3.4 g, 25 mmol), compound **2** (7.1 g, 25 mmol) and KOH (1.4 g, 25 mmol) were dissolved in MeOH (100 ml). The reaction mixture was treated as in general method for the synthesis of chalcone to give **11** as yellow needles (2.3 g, 5.7 mmol, 23%). IR (KBr) 1648, 1586, 1508, 1492, 1249, 1222, 1171 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.81 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 4.73 (2H, dd, $J = 6.0$, 1.6 Hz, OCH_2), 6.41 (H, dt, $J = 15.6$, 6.0 Hz, H-2″), 6.75 (1H, d, $J = 16$ Hz, H-α), 6.94 (1H, d, $J = 9.2$ Hz, H-3′), 6.96 (2H, dd, $J = 8.8$, 2.8 Hz, H-3 and H-5), 7.00 (1H, dd, $J = 8.8$, 2.8 Hz,

H-4′), 7.17 (1H, d, $J = 2.8$ Hz, H-6′), 7.25–7.40 (6H, m, H-3″–H-9″), 7.55 (2H, dd, $J = 8.8$, 2.8 Hz, H-2 and H-6), 7.61 (1H, d, $J = 16$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 55.8 (OCH_3), 56.5 (OCH_3), 68.7 (C-1″), 113.4 (C-6′), 114.3 (C-3′), 115.1 (C-2 and C-6), 118.8 (C-α), 123.8 (C-2″), 124.8 (C-4′), 126.6 (C-5″ and C-9″), 128.0 (C-1″ and C-7″), 128.6 (C-6″ and C-8″), 130.0 (C-1), 130.2 (C-2 and C-6), 133.4 (C-3″), 136.2 (C-4″), 143.4 (C-β), 152.4 (C-5′), 154.6 (C-2′), 160.5 (C-4), 192.6 (C=O); MS (EI) *m/z* 400 (M^+, 1). Anal Calcd for $C_{26}H_{24}O_4$: C, 78.00; H, 6.00. Found: C, 77.89; H, 6.1.

*5.1.10. 2′,5′-Dimethoxy-4-(4-methylcinnamoyloxy)chalcone (**12**)*

4-Methylcinnamic acid (3.2 g, 25 mmol) in anhydrous C_6H_6 (60 ml) was treated as in general method for the synthesis of chalcone to give **12** as yellow needles (4.38 g, 10.25 mmol, 41%). IR (KBr) 1733, 1630, 1594, 1578, 1491, 1212, 1165, 1132 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 2.39 (3H, s, CH_3), 3.80 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.58 (1H, d, $J = 16.0$ Hz, H-α), 6.94 (1H, d, $J = 8.8$ Hz, H-3′), 7.02 (1H, dd, $J = 9.2$, 2.8 Hz, H-4′), 7.22 (5H, m, H-6′ and H-2–6), 7.40 (1H, d, $J = 15.6$ Hz, H-2″), 7.48 (2H, d, $J = 8.0$ Hz, H-6″ and H-8″), 7.61 (2H, dd, $J = 8.0$, 2.0 Hz, H-5″ and H-9″), 7.65 (1H, d, $J = 15.6$ Hz, H-3″), 7.86 (1H, d, $J = 16.0$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 21.4 (CH_3), 55.7 (OCH_3), 56.4 (OCH_3), 113.3 (C-6′), 114.3 (C-3′), 115.6 (C-2″), 119.2 (C-α), 122.1 (C-3 and C-5), 126.8 (C-4′), 128.3 (C-6″ and C-8″), 129.4 (C-1′, C-2 and C-6), 129.7 (C-5″ and C-9″), 131.2 (C-1), 132.7 (C-4″), 141.3 (C-7″), 142.0 (C-β), 147.0 (C-3″), 152.2 (C-4), 152.5 (C-4′), 153.5 (C-2′), 165.2 (C-1″), 192.1 (C=O); MS (EI) *m/z* 428 (M^+, 1). Anal Calcd for $C_{27}H_{24}O_5$: C, 75.70; H, 5.60. Found: C, 75.61; H, 5.71.

*5.1.11. 2′,5′-Dimethoxy-4-(4-methoxycinnamoyloxy)-chalcone (**13**)*

4-Methoxycinnamic acid (4.5 g, 25 mmol) in anhydrous C_6H_6 (60 ml) was treated as in general method for the synthesis of chalcone to give **13** as yellow needles (4.45 g, 10.03 mmol, 40.1%). IR (KBr) 1718, 1654, 1629, 1601, 1508, 1166, 1131 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.81 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.49 (1H, d, $J = 15.6$ Hz, H-α), 6.94 (1H, d, $J = 8.8$ Hz, H-3′), 6.95 (2H, dd, $J = 8.8$, 2.8 Hz, H-3 and H-5), 7.02 (1H, dd, $J = 8.8$, 2.8 Hz, H-4′), 7.20 (1H, d, $J = 2.8$ Hz, H-6′), 7.22 (2H, dd, $J = 8.8$, 2.8 Hz, H-2 and H-6), 7.39 (1H, d, $J = 16.0$ Hz, H-3″), 7.53 (2H, dd, $J = 8.8$, 2.0 Hz, H-6″ and H-8″), 7.61 (2H, dd, $J = 8.8$, 2.0 Hz, H-5″ and H-9″), 7.64 (1H, d, $J = 16.0$ Hz, H-2″), 7.82 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 55.4 (OCH_3), 55.8 (OCH_3), 56.5 (OCH_3), 113.4 (C-6′), 114.2 (C-3′), 114.4 (C-2″, C-6″ and C-8″), 119.3 (C-α), 122.2 (C-3 and C-5), 126.8 (C-4″), 126.9 (C-4′), 129.5 (C-1′), 129.6 (C-2 and C-6), 130.1 (C-5″ and C-9″), 132.7 (C-1), 142.1 (C-β), 146.7 (C-3″), 152.4 (C-4), 152.6 (C-4′), 153.6 (C-2′), 161.8 (C-7″), 165.4 (C-1″), 192.2 (C=O); MS (EI) *m/z* 444 (M^+, 1). Anal Calcd for $C_{27}H_{24}O_6$: C, 73.00; H, 5.40. Found: C, 72.90; H, 5.45.

*5.1.12. 2′,5′-Dimethoxy-4-(3,4,5-trimethyoxy cinnamoyloxy)-chalcone (**14**)*

3,4,5-Trimethoxycinnamic acid (6.0 g, 25 mmol) in anhydrous C_6H_6 (60 ml) was treated as in general method for the synthesis of chalcone to give **14** as yellow needles (3.4 g, 25 mmol, 11%). IR (KBr) 1726, 1635, 1581, 1503, 1273, 1210, 1166, 1127 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 3.79 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 3.89 (3H, s, OCH_3), 3.89 (6H, s, OCH_3), 6.53 (1H, d, $J = 15.6$ Hz, H-α), 6.81 (2H, s, H-5″ and H-9″), 6.92 (1H, d, $J = 9.2$ Hz, H-3′), 7.00 (1H, dd, $J = 9.2$, 3.2 Hz, H-4′), 7.18 (1H, d, $J = 3.2$ Hz, H-6′), 7.18 (1H, d, $J = 3.2$ Hz, H-6′), 7.20 (2H, dd, $J = 9.2$, 3.2 Hz, H-3 and H-5), 7.38 (2H, d, $J = 16.0$ Hz, H-2″), 7.62 (2H, dd, $J = 9.2$, 3.2 Hz, H-2 and H-6), 7.63 (1H, d, $J = 16.0$ Hz, H-3″), 7.78 (1H, d, $J = 15.6$ Hz, H-β). ^{13}C NMR ($CDCl_3$): δ 55.7 (OCH_3), 56.1 (OCH_3), 56.4 (OCH_3), 60.9 (OCH_3), 105.4 (C-5″ and C-9″), 113.3 (C-6′), 114.3 (C-3′), 116.0 (C-2″), 119.2 (C-α), 122.0 (C-3 and C-5), 126.9 (C-4′), 129.4 (C-1′, C-6 and C-4″), 132.7 (C-1), 141.9 (C-β), 146.9 (C-3″), 152.1 (C-4), 152.5 (C-5′), 153.4 (C-2′, C-6″ and C-8″), 165.0 (C-1″), 192.0 (C=O); MS (EI) *m/z* 504 (M^+, 1). Anal Calcd for $C_{29}H_{28}O_8$: C, 69.00; H, 5.60. Found: C, 68.81; H, 5.63.

5.2. Tumor cell growth inhibition assays

The cytotoxicity of compounds **3–14** against human lung cancer cell A549, human hepatomacellular carcinoma Hep 3B, human colerectal adenocarcinoma HT-29 and human breast adenocarcinoma MCF-7 cells obtained from American Type Culture Collection (ATCC, Rockville, MD), were performed by the method as described in Ref. [5].

5.3. Flow cytometry

Compounds were added to cells (1×10^7). At various time intervals, the reactions were terminated by washing with PBS. The cells were fixed with 4% paraformadehyde/PBS (pH 7.4) at room temperature for 30 min. After centrifugation at 1000 rpm for 10 min, the cells were permeabilized with 0.1% Triton-X-100/0.1% sodium citrate at 4 °C for 2 min. Propidium iodide (Sigma) in PBS (10 g/mL) was added to stain the cells at 37 °C for 30 min. The intensity of fluorescence was measured with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). A minimum of 5000 cell counts were collected for the analysis by LYSIS II Software.

5.4. Microscopic observation of morphology and nuclear fragmentation

Morphological changes of the cells were observed directly in culture vehicles. To observe chromatin condensation and plasma membrane permeability, cell were resuspended in fresh culture medium after collection by centrifugation, co-stained propidium iodide (PI, 0.05 g/L) and Hoechst 33258 (0.01 g/L) at 37 °C for 30 min, then centrifuged to remove most

of the supernatant. The pellet was suspended in the remaining culture medium and smeared for fluorescence microscope.

5.5. Mast cell degranulation, neutrophil degranulation, superoxide anion generation macrophage cultures and drugs treatment and NO determination

The inhibitory assays for chemical mediator induced by various stimulants in mast cells, neutrophils, and RAW 264.7 cells were performed by the methods as described in Ref. [16].

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