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Short communication

Synthetic chalcones as potential anti-inflammatory and cancer chemopreventive agents

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Abstract

In an effort to develop potent anti-inflammatory and cancer chemopreventive agents, a series of chalcones were prepared by Claisen–Schmidt condensation of appropriate acetophenones with suitable aromatic aldehyde or prepared with appropriate dihydrochalcone reacted with appropriate alkyl bromide or prepared in one-pot procedure involving acetophenone and convenient aromatic aldehyde using ultrasonic agitation on basic alumina. The synthesized products were tested for their inhibitory effects on the activation of mast cells, neutrophils, macrophages, and microglial cells. The potent inhibitors of NO production in macrophages and microglial cells were further evaluated for their in vitro cytotoxic effects against several human cancer cell lines. 2′-Hydroxychalcones **1**–**3**, and 2′,5′-dihydroxychalcone **7** exhibited potent inhibitory effects on the release of β-glucuronidase or lysozyme from rat neutrophils stimulated with formyl-Met-Leu-Phe (fMLP)/cytochalasin B (CB). Two 2′-hydroxychalcones (**1** and **3**) showed potent inhibitory effects on superoxide anion generation in rat neutrophils in response to fMLP/CB. The previously reported chalcone, **5**, **6**, and **12**, exhibited potent inhibitory effect on NO production in lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-activated N9 microglial cells or in LPS-activated RAW 264.7 macrophage-like cells. The potent inhibitors **5**, **6**, and **12** of NO production in macrophages or microglial cells revealed significant or marginal cytotoxic effects against several human cancer lines. Compound **12** manifested potent selective cytotoxicity against human MCF-7 cells and caused cell death by apoptosis. The present results demonstrated that **1**–**3**, and **7** have anti-inflammatory effects and **5**, **6**, and **12** are potential anti-inflammatory and cancer chemopreventive agents.

Keywords: Chalcone; Anti-inflammatory; Cancer chemopreventive agent

1. Introduction

It is conceivable that mast cells, neutrophils, and macrophages are important players in inflammatory disorders. Activation of microglial cells also plays a crucial role in inflammatory diseases of the CNS. Thus, inhibition of the activation of these inflammatory cells appears to be an important therapeutic target for small molecule drug design for the treatment of inflammatory diseases. NO plays a central role in macrophage-induced cytotoxicity and expressed NO may contribute to the pathophysiology of septic shock [1]. The excessive production of NO also can destroy functional normal tissues during acute and chronic inflammation [2]. This phenomenon is also closely related mechanistically to carcinogenesis [2]. Thus, inhibitors of NO production in macrophages are potential anti-inflammatory and cancer chemopreventive drugs.

Our recent reports have demonstrated that some synthetic chalcones inhibited the release of chemical mediators from mast cells, neutrophils, macrophages, and microglial cells in vitro, and suppressed the oedematous response in vivo [3–6]. We also reported that broussochalcone A, a natural chalcone

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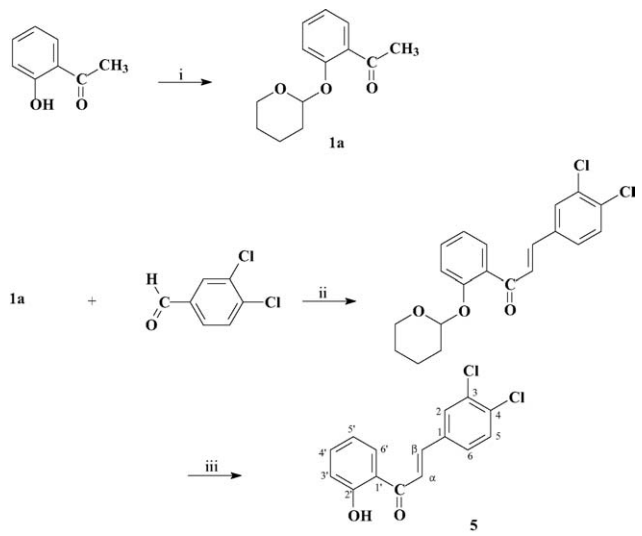
isolated from *Broussonetia papyrifea* (Moraceae), exerts a potent antioxidant activity and inhibits the respiratory burst in neutrophils and the inducible nitric oxide synthase (iNOS) expression in macrophages [7,8]. Moreover, two synthetic products, 2′,5′-dihydroxy-4-chloro-dihydrochalcone and 2′,5′-dihydroxydihydrochalcone inhibit the iNOS protein expression and the former also inhibits cyclooxygenase-2 (COX-2) activity in RAW 264.7 cells [6,9].

Chalcones constitute an important group of natural products and some of them posses antitumor activity [10]. These findings suggested that some chalcones may be promising anti-inflammatory and cancer chemopreventive agents and have potential in the therapy of septic shock. This report described the chemistry of further synthesized chalcones, biological activity, and the structure–activity relationships of these series of anti-inflammatory and cancer chemopreventive agents.

2. Chemistry

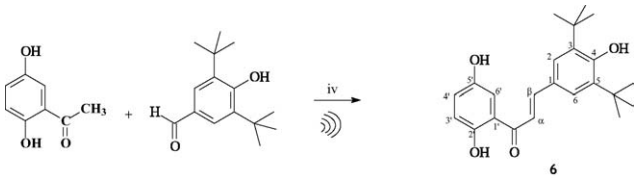
As depicted in Scheme 1 for the specific synthesis of compound **5**, in addition to the new chalcones **3**, **5**–**7**, we have prepared compounds **1** [3,11–17], **2** [18–22], **4** [19,20,23,24], the 3,4-dichloro-2′,5′-dimethoxychalcone (**8a**), 3,4-dichloro-2′,5′-dihydroxychalcone (**9a**) [4,6] and the 4-chloro-2′,5′-dihydroxychalcone (**10a**) [4,26] by carrying out Claisen–Schmidt condensations starting from appropriate tetrahydropyran-2-yloxyacetophenones and requisite heterocyclic aldehydes or aromatic aldehydes (protected as tetrahydropyran-2-yl ethers if necessary) [4]. This procedure afforded various chalcones in good yield (Table 1). A solution **8a**, **9a**, or **10a** was hydrogenated in the presence of Pd/C at room temperature to give the new dihydrochalcones **8** and **9** [5], respectively. The reduced product: 4-chloro-2′,5′-dihydroxydihydrochalcone (**10b**) and anhydrous K_2CO_3 were stirred with 3-bromo-1-propene in *N*,*N*-dimethylformamide (DMF) at room temperature to give the novel dihydrochalcone **10**. Compound **10b** was also refluxed with 3-bromo-1-butene (Scheme 3). In this latter case, the subsequent Claisen rearrangement gave **11′**. The occurrence of secondary Claisen rearrangement give the new dihydrochalcone, **11** [26]. The ^{1}H-NMR and ^{13}C-NMR spectra (Section 5) supported the structure of **11**. One-pot synthesis involving the appropriate acetophenone and the suitable aromatic aldehyde by ultrasonic agitation on basic alumina provided the novel chalcone **6** (Scheme 2). This is the first report dealing with a convenient one-pot synthesis of chalcones by ultrasonic agitation on basic alumina [27].



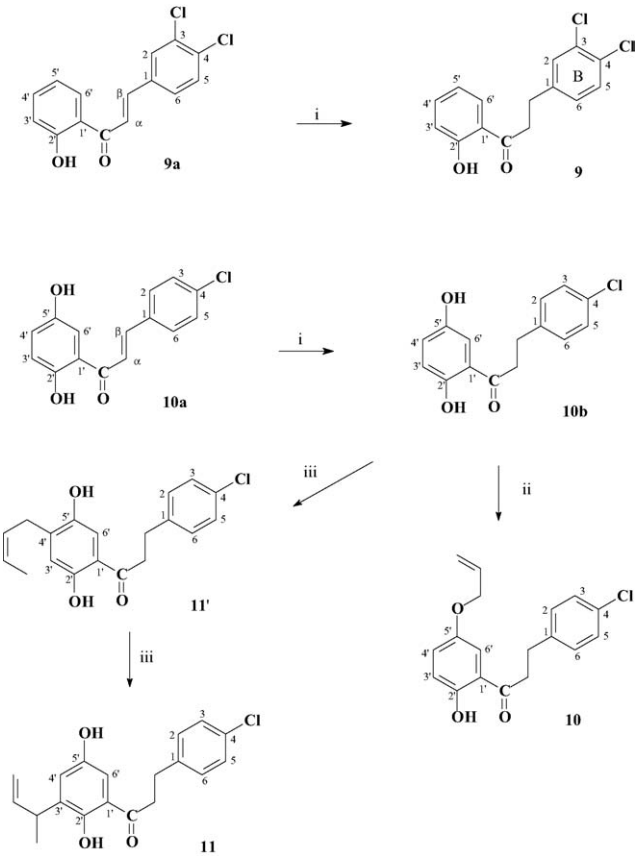
Scheme 1. Reagents : (i) pyridium *p*-toluenesulfonate, 3,4-dihydro-α-pyran, r.t., 4 h ; (ii) $BaOH{\cdot}8H_2O$, 40 °C, HCl ; (iii) *P*-toluenesulfonic acid, r.t., 4 h, 5 % $NaHCO_3$.

3. Biological results and discussion

The anti-inflammatory activities of **1**–**11** were studied in vitro for their inhibitory effects on chemical mediators re-

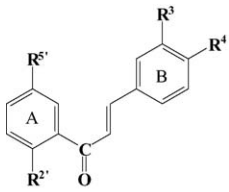


Scheme 2. Reagents: (iv) Al_2O_3, 40 °C, 12 h.

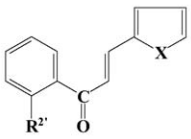


Scheme 3. Reagents: (i) EtOAc, H_2, 5% Pd/C, r.t.; (ii) 3-bromo-1-propene, K_2CO_3, DMF, r.t., 18 h; (iii) 3-bromo-1-butene, K_2CO_3, DMF, reflux 18 h.

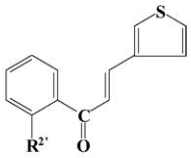
Table 1
Structure and analytical data of chalcone derivatives



1, 5, 12



2 : X=S, 4 : X=O



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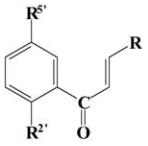
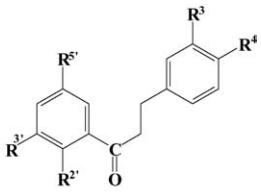


6, 7



8-11

Compd	$R^{2'}$	$R^{3'}$	$R^{5'}$	R^3	R^4	R	formula	mp (°C)	Yield (%)	analyses[a]
1	OH	H	H	H	OH		$C_{15}H_{12}O_3$	158-160	41[b]	C, H
2	OH	H	H	H	H		$C_{13}H_{10}O_2S$	99-101	53[b]	C, H
3	OH	H	H	H	H		$C_{13}H_{10}O_2S$	74-76	51[b]	C, H
4	OH	H	H	H	H		$C_{13}H_{10}O_3$	106-108	41[b]	C, H
5	OH	H	H	Cl	Cl		$C_{15}H_{10}Cl_2O_2$	150-152	58[b]	C, H
6	OH	H	OH	H	H		$C_{23}H_{28}O_4$	72-74	10[b]	C, H
7	OH	H	OH	H	H		$C_{17}H_{13}NO_3$	212-214	23[b]	C, H, N
8	OMe	H	OMe	Cl	Cl		$C_{17}H_{16}Cl_2O_3$	81-83	50[b]	C, H
9	OH	H	OH	Cl	Cl		$C_{15}H_{12}Cl_2O_3$	165-167	43[b]	C, H
10	OH	H		H	Cl		$C_{18}H_{17}ClO_3$	oil	11	C, H
11	OH	CH_2=CH-CH(CH_3)-	OH	H	Cl		$C_{19}H_{19}ClO_3$	oil	7	C, H
12[c]	OCH_3	H	OCH_3	H	OH		$C_{17}H_{16}O_4$	127-128	48[b]	C, H

[a]C, H and N analyses were within ± 0.4 % of the theoretical values. Solvents used for recrystallization: [b]$CHCl_3$. [c]Data cited from Ref. 6.

leased from mast cells, neutrophils, macrophages, and microglial cells. Compounds **1**–**11** did not show significant inhibition of mast cell degranulation (data not shown). Previously, we reported that 2′,5′-dihydroxy-3,4-dichlorochalcone showed inhibition of mast cell degranulation [6], while **5** and **9** did not indicate significant inhibition of mast cell degranulation. It shows that the OH-5′ and enone moieties of 2′,5′-dihydroxy-3,4-dichlorochalcone appear to be required for the inhibition of mast cell degranulation. 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 **2**, **3**, and **7**, and **1**–**3** had potent and concentration-dependent inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils, respectively (Table 2). Based on the data appeared on the Table 2, it clearly indicates that the OH-5′ enhances the inhibitory effects on the release of β-glucuronidase and lysozyme from rat neutrophils in the series of 2′-hydroxy-3,4-dichloro or 2-furfuryl or 2- or 3-thienylchalcone [4,6]. In addition, the essential role of the enone moiety of chalcones in the inhibition of fMLP/CB-stimulated neutrophil degranulation reconcile our earlier observation [5]. The B ring of 2′,5′-dihydroxychalcone substituted by a indole ring such as **7** (Table 2) showed potent and concentration-dependent inhibition effect on the release of β-glucurnidase from rat neutrophils. Trifluoperazine was used in this study as a positive control.

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 3, compounds **1** and **3** had potent and concentration-dependent inhibitory effects on fMLP/CB-induced superoxide anion generation. Based on the data shown on Table 3, it indicates that a hydroxyl group substituted at C-5′ of **2** or **3** does not enhance the inhibitory effect on fMLP/CB-induced superoxide anion generation in

Table 2
The inhibitory effects of chalcone derivatives on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP/CB

Compound	IC_{50} (μM) [a]	
	β-glucuronidase	Lysozyme
1	[b]	7.5 ± 0.3
2	23.5 ± 1.4	27.4 ± 0.7
3	8.7 ± 0.7	13.4 ± 2.5
4	>30 (35.1 ± 2.8)	>30 (17.7 ± 12.2)
5	>30 (21.8 ± 3.6)	>30 (15.0 ± 0.9)
6	>3 (33.7 ± 4.7)	>3 (14.8 ± 2.0)
7	17.1 ± 4.2	>30 (45.7 ± 6.5)
8	>30 (9.4 ± 3.5)	>30 (−3.6 ± 2.4)
9	>30 (41.5 ± 2.8)	>30 (22.6 ± 0.5)
10	>30 (9.2 ± 0.5)	>30 (2.0 ± 3.3)
11	>3 (2.7 ± 5.4)	>3 (−5.5 ± 3.1)
13*	17.3 ± 4.1 [c]	19.7 ± 5.0 [c]
14*	9.7 ± 1.9 [c]	23.6 ± 4.9 [c]
15*	4.6 ± 0.4 [d]	6.7 ± 0.3 [d]
16*	1.3 ± 0.1 [d]	1.2 ± 0.1 [d]
Trifluoperazine	12.2 ± 0.3	13.2 ± 0.7

13*: 2′,5′-Dihydroxy-2-furfurylchalcone. **14***: 2′,5′-Dihydroxy-2-thienylchalcone. **15***: 2′,5′-Dihydroxy-3-thienylchalcone. **16***: 2′,5′-Dihydroxy-3,4-dichlorochalcone. Trifluoperazine was used as a positive control.

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses.

[b] Not determined. Data are presented as mean ± S.E.M. (n = 3–5).

[c] Data cited from Ref. [4].

[d] Data cited from Ref. [6].

Table 3
The inhibitory effects of chalcone derivatives on superoxide anion generation in rat neutrophils stimulated with fMLP/CB or PMA

Compound	IC_{50} (μM) [a]	
	fMLP	PMA
1	6.4 ± 0.3	>30 (35.5 ± 2.5)
2	>30 (41.8 ± 9.8)	>30 (8.4 ± 4.6)
3	8.2 ± 0.3	>30 (32.0 ± 6.9)
4	>30 (39.2 ± 12.2)	>30 (17.6 ± 1.5)
5	>30 (46.1 ± 7.4)	>30 (24.7 ± 6.0)
6	>1 (0.1 ± 7.6)	>1 (12.7 ± 9.5)
7	>1 (16.1 ± 1.9)	>1 (−3.1 ± 4.2)
8	>30 (−42.4 ± 4.6)	>30 (6.9 ± 4.2)
9	>30 (−6.0 ± 5.1)	>30 (−12.6 ± 3.4)
10	>30 (−7.0 ± 1.9)	>30 (−19.8 ± 2.2)
11	>10 (−1.1 ± 5.7)	>10 (−74.7 ± 8.2)
14	>100 (−10.8 ± 8.5) [b]	24.3 ± 4.5 [b]
15	>1 (43.4 ± 6.1) [c]	>1 (23.5 ± 10.4) [c]
Trifluoperazine	8.3 ± 0.8	6.1 ± 0.4

Data are presented as mean ± S.E.M. (n = 3–5). Trifluoperazine was used as a positive control.

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses.

[b] Data cited from Ref. [4].

[c] Data cited from Ref. [6].

rat neutrophils while a hydroxyl group substituted at C-5′ of **2** does enhance the inhibitory effect on PMA-induced superoxide anion generation [4,6]. Because fMLP and PMA activate NADPH oxidase to produce superoxide anion through different cellular signaling mechanisms [28]. The observations of **1–11** had no appreciable effect on PMA-induced response suggest the involvement of PMA-independent signaling pathway. In addition, the essential role of the enone moiety of chalcones in the inhibition of fMLP/CB- or PMA-stimulated superoxide anion generation also reconcil on earlier observation [6].

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 reaction. As shown in Table 4, **5**, **6**, and 2′,5′-dimethoxy-4-hydroxychalcone (**12**), a previously reported compound [6], showed potent and concentration-dependent inhibitory effects on NO accumulation from RAW 264.7 cells. The parallel inhibition of NO production in N9 microglial cells as well as in RAW 264.7 cells by **5** and **12**. The dihydrochalcones, **8–11** (Table 4) did not show significant inhibitory effects on NO accumulation from RAW 264.7 stimulated with LPS and N9 microglial cells stimulated with LPS/interferon-γ (IFN-γ), while 2′,5′-dihydroxydihydrochalcone (**17**) showed potent inhibitory effect on NO production from RAW 264.7 cells [5]. It suggests that **5**, **12**, and **17** may be the potential leading compounds for the development of more potent drugs to inhibit the NO production in macrophages or microglial cells.

Cytotoxic activities of compounds **3**, **5**, **6**, and **12** with potent inhibition of NO production from RAW 264.7 cells activated with LPS except that **3** did not show significant inhibition of NO production, were studied against a number of cancer cell types. The results were listed in Table 5. All compounds in Table 5 showed selective cytotoxicity against MCF-7 cells. The lipophilicity of A or B ring did affect the

Table 4
The inhibitory effects of chalcone derivatives on the accumulation of NO_2^- in the culture media of RAW 264.7 cells in response to LPS and N9 cells in response to LPS/IFN-γ

Compound	IC_{50} (μM) [a]	
	RAW 264.7 cells	N9 cells
1	>30 (49.5 ± 1.9)	>10 (7.3 ± 0.8)
2	>30 (13.5 ± 2.1)	>30 (12.7 ± 2.3)
3	>30 (−4.8 ± 0.4)	>30 (20.4 ± 1.2)
4	>30 (23.2 ± 2.7)	>30 (18.8 ± 2.3)
5	23.8 ± 1.0	11.8 ± 0.2
6	23.3 ± 2.6	>10 (42.3 ± 1.6)
7	>10 (33.6 ± 0.6)	>10 (15.8 ± 1.1)
8	>30 (3.3 ± 1.7)	>10 (28.7 ± 2.2)
9	>1 (27.0 ± 0.1)	>3 (43.7 ± 1.4)
10	>30 (−1.0 ± 0.4)	>30 (12.5 ± 1.2)
11	>30 (30.3 ± 2.9)	>10 (1.3 ± 8.1)
12	14.6 ± 0.1 [c]	17.8 ± 0.2 [b]
17*	11.8 ± 0.9 [c]	>3 (48.8 ± 3.3) [c]
1400 W	6.1 ± 0.4	4.5 ± 0.2

Data are presented as mean ± S.E.M. (n = 3–5). 1400 W [*N*-(3-aminomethyl)benzyl-acetamidine] was used as a positive control. **17***: 2′,5′-Dihydroxydihydrochalcone.

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses.

[b] Data cited from Ref. [6].

[c] Data cited from Ref. [5].

Table 5
Cytotocixity of chalcone derivatives against different cell lines [a]

Compound	ED_{50} (μg ml^{-1})			
	T-24	MCF-7	Hep 3B	HT-29
3	>8	2.10	>8	>8
5	2.98	2.12	3.00	4.00
6	7.40	3.60	7.10	9.00
12	>4	0.16	2.00	4.60
5-Fu	[b]	[b]	0.072	0.074
Mitomycin C	0.042	[b]	[b]	[b]

[a] For significant activity of the pure compounds, an $ED_{50} \leq 4.0$ μg ml^{-1} is required.
[b] Not determined. 5-Fu (5-fluorouracil) was used as a positive control.

cytotoxicity of chalcones against the cell lines used in Table 5.

It has been recognized that apoptotic cells have reduced DNA stainability with a variety of fluorochromes [29,30]. The appearance of cells with low DNA stainability forms a "sub-G_1 peak", which has been considered to be the hallmark of cell death by apoptosis [31]. In the study, various concentrations of **12** 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 **12** at various concentrations for different time periods. The shift of G_0/G_1 and G_2/M cell cycles to the sub-G_1 phase is increased dose-dependently in the MCF-7 cells treated by **12** for different time periods. However a maximum 65.88% apoptotic cells was detected at 72 h. In addition, DNA fragmentation is generally used to characterize cell death by apoptosis [32,33]. Thus, apoptosis of the MCF-7 cells after **12** treatment was also studied by DNA fragmentation. DNA fragmentation in MCF-7 cells was significantly observed after 48 and 72 h incubation with **12** (1.0 and 2.0 μg ml^{-1}; Fig. 2). Further experiments are needed to elucidate its mechanism of action.

4. Conclusions

This study verifies that 2′-hydroxychalcones **1**–**3**, 2′,5′-dihydroxychalcone **7** exert potent inhibitory effects on the release of chemical mediators from inflammatory cells. NO plays a central role in macrophage-induced cytotoxicity and has been demonstrated to implicate in the pathology of central neurologic diseases and also in the peripheral tissue damage associated with acute and chronic inflammation [6,34,35], and septic shock [1]. The present study suggests that the inhibition of NO production by **5** and **6** in macroph-

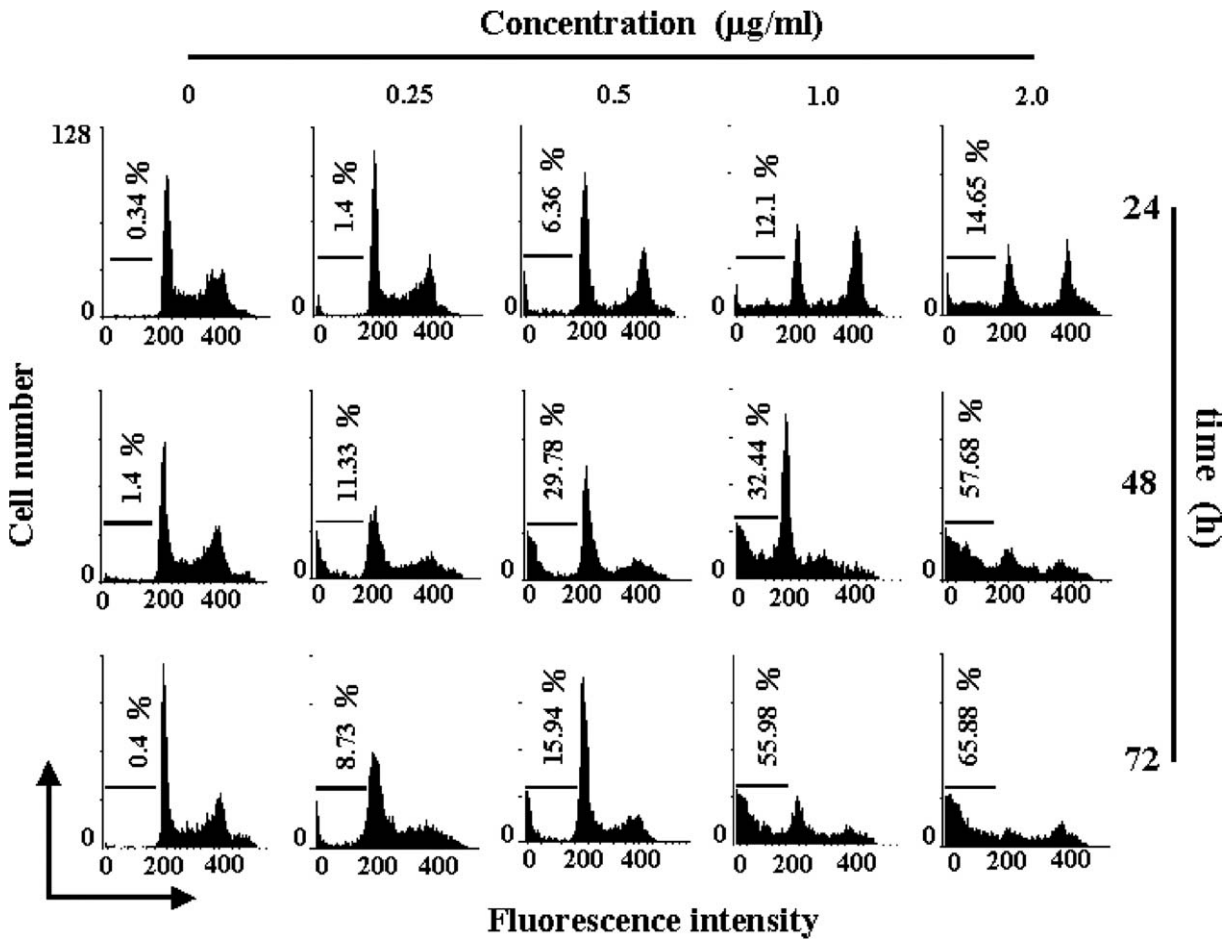


Fig. 1. Flow cytometry analysis of **12** treated MCF-7 cells. MCF-7 (1×10^4 cells ml^{-1}) were treated with various concentrations of **12** for different time periods. At the times indicated, cells were stained with propidium iodide (PI), and DNA contents were analyzed by flow cytometry. Apoptosis was measured by the accumulation of sub-G1 DNA contents in cells. The control cells were treated with medium. Results were representative of three independent experiments.

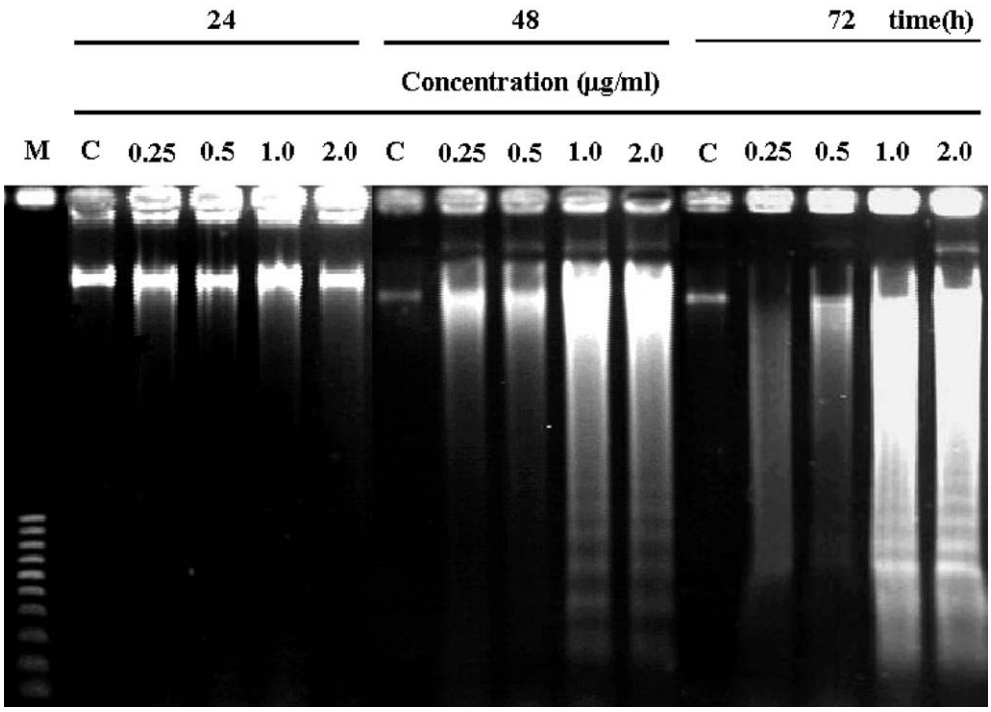


Fig. 2. Compound **12** induces DNA fragmentation in MCF-7 cells. MCF-7 (1×10^4 cells ml^{-1}) cells were treated with various concentrations of **12** for different time periods. At the times indicated, the cells were lyzed and DNA was prepared. DNA fragmentation was analyzed by 1% agarose gel electrophoresis. The compound **12** was diluted with culture medium. The control cells were treated with medium. M, DNA molecular ladder marker. Results were representative of three independent experiments.

ages and **5** in microglial cells may have value in the therapeutic treatment or prevention of certain central as well as peripheral inflammatory diseases associated with the increase of NO production. The inhibition of NO production by **5**, **6**, and **12** in macrophages and significant cytotoxic activities by **5**, **6**, and **12** against the cell lines used in Table 5 suggest that **5**, **6**, and **12** may be used as cancer chemopreventive drugs [2]. Thus, compound such **12** has the potential to develop as cancer chemopreventive drug for treatment or prevention of MCF-7 cell type cancer.

5. Experimental protocols

Melting points (uncorrected) were determined with a Yanaco Micro-Melting Point apparatus. IR spectra were determined with a Perkin–Elmer system 2000 FTIR spectrophotometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Varian Unity-400 spectrometer, and Mass were obtained on a JMS-HX 100 mass spectrometer. Elemental analyses were within ±0.4% of the theoretical values, unless otherwise noted. Chromatography was performed using a flash-column technique on silica gel 60 supplied by E. Merck.

Chalcone **12** has been synthesized as previously reported [6].

General procedure for obtaining chalcones **1**–**5**, **7**, 3,4-dichloro-2′,5′-dimethoxy-chalcone (**8a**), 3,4-dichloro-2′,5′-dihydroxychalcone (**9a**), and 4-chloro-2′,5′-dihydroxychalcone (**10a**) [4].

5.1. 2′,4-Dihydroxychalcone (1)

2-Hydroxyacetophenone (3.4 g, 25 mmol) or 4-hydroxybenzaldehyde (3.05 g, 25 mmol) and pyridinium *p*-toluenesulfonate (0.15 g, 0.6 mmol) were treated as in the synthesis of 2′,3-dihydroxychalone to give 2-(tetra-hydropyran-2-yloxy)acetophenone (**1a**) or 4-(tetrahydroxypyran-2-yloxy)benzyl-aldehyde (**1b**) [4]. The **1a** or **1b** was identified with various spectral data and compared with those of authentic samples, respectively [4,6]. Compound **1a** (5.5 g, 25 mmol), **1b** (5.15 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of 2′,3-dihydroxychalcone to give **1** (2.47 g, 10.3 mmol, 41%): IR (KBr) 3347, 1634, 1610, 1580 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 6.89 (2H, dd, J = 8.2, 1.4 Hz, H-3 and 5), 6.96 (1H, dd, J = 8.2, 2.0 Hz, H-3′), 7.03 (1H, td, J = 8.2, 2.0 Hz, H-5′), 7.50 (2H, dd, J = 8.2, 1.4 Hz, H-2 and 6), 7.54 (1H, d, J = 15.2 Hz, H-α), 7.59 (1H, td, J = 8.2, 2.0 Hz, H-4′), 7.89 (1H, d, J = 15.2 Hz, H-β), 7.92 (1H, dd, J = 8.2, 2.0 Hz, H-6′), 12.94 (1H, s, OH-2′). ^{13}C-NMR ($CDCl_3$): δ 118 (C-3 and 5), 119.8 (C-3′), 120.5 (C-α), 129.2 (C-2 and 6), 129.5 (C-5′), 129.7 (C-1′), 133.0 (C-6′), 136.4 (C-4′), 136.8 (C-1′), 143.8 (C-β), 163.5 (C-2′ and 4), 193.3 (C=O). EIMS (70 eV) *m*/*z* (% rel. int.): 240 (75) $[M]^+$.

*5.2. 2′-Hydroxy-2-thienylchalcone **(2)***

Compound **1a** (5.5 g, 25 mmol), 2-thiophen-aldehyde (2.8 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **2** (3.06 g, 13.3 mmol, 53%): IR (KBr) 3484, 1636, 1560 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 6.95 (1H, td, J = 8.2, 1.0 Hz, H-5′), 7.03 (1H, dd, J = 8.2, 1.4 Hz, H-3′), 7.39 (1H, d, J = 3.6 Hz, H-3), 7.43 (1H, dd, J = 4.3, 3.6 Hz, H-4), 7.51 (1H, td, J = 8.2, 1.4 Hz, H-4′), 7.61 (1H, d, J = 4.3 Hz, H-5), 7.62 (1H, d, J = 15.4 Hz, H-α), 7.86 (1H, d, J = 15.4 Hz, H-β), 7.91 (1H, dd, J = 8.2, 1.4 Hz, H-6′), 12.70 (1H, s, OH-2′). ^{13}C-NMR ($CDCl_3$): δ 118.5 (C-3′), 118.7 (C-α), 119.8 (C-1′), 128.4 (C-5′), 129.4 (C-3 and 6′), 132.6 (C-4), 136.2 (C-4′ and 5), 137.7 (C-β), 140.0 (C-2), 193.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 230 (74) $[M]^+$.

*5.3. 2′-Hydroxy-3-thienylchalcone **(3)***

Compound **1a** (5.5 g, 25 mmol), 3-thiophen-aldehyde (2.8 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **3** (2.94 g, 12.8 mmol, 51%): IR (KBr) 3498, 1639, 1560 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 6.91 (1H, td, J = 8.4, 1.5 Hz, H-5′), 7.01 (1H, dd, J = 8.4, 1.5 Hz, H-3′), 7.35 (1H, dd, J = 5.1, 2.7 Hz, H-5), 7.41 (1H, dd, J = 5.1, 1.4 Hz, H-4), 7.43 (1H, d, J = 15.1 Hz, H-α), 7.48 (1H, dd, J = 8.4, 1.5 Hz, H-4′), 7.60 (1H, dd, J = 2.7, 1.4 Hz, H-2), 7.86 (1H, dd, J = 8.4, 1.5 Hz, H-6′), 7.88 (1H, d, J = 15.1 Hz, H-β), 12.90 (1H, s, OH-2). ^{13}C-NMR ($CDCl_3$): δ 118.4 (C-2), 118.6 (C-3′), 119.5 (C-α), 119.8 (C-1′), 125.0 (C-5′), 127.0 (C-4), 129.4 (C-4′), 129.8 (C-5), 136.1 (C-6′), 137.7 (C-3), 138.6 (C-β), 163.3 (C-2′), 193.7 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 230 (100) $[M]^+$.

*5.4. 2′-Hydroxy-2-furfurylchalcone **(4)***

Compound **1a** (5.5 g, 25 mmol), 2-furaldehyde (2.4 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **4** (2.18 g, 10.2 mmol, 41%): IR (KBr) 3425, 1639, 1582 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 6.52 (1H, dd, J = 3.4, 1.8 Hz, H-4), 6.75 (1H, d, J = 3.4 Hz, H-3), 6.92 (1H, td, J = 8.5, 1.6 Hz, H-5′), 7.01 (1H, dd, J = 8.5, 1.6 Hz, H-3′), 7.48 (1H, td, J = 8.5, 1.6 Hz, H-4′), 7.53 (1H, d, J = 15.0 Hz, H-α), 7.55 (1H, d, J = 1.8 Hz, H-5), 7.57 (1H, d, J = 15.0 Hz, H-β), 7.90 (1H, dd, J = 8.5, 1.6 Hz, H-6′), 12.90 (1H, s, OH-2′). ^{13}C-NMR ($CDCl_3$): δ 112.8 (C-3), 117.0 (C-4), 117.5 (C-3′), 118.4 (C-5′), 118.7 (C-α), 119.9 (C-1′), 129.5 (C-4′), 131.0 (C-6′), 136.2 (C-5), 145.3 (C-β), 151.4 (C-2), 163.4 (C-2′), 193.2 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 214 (53) $[M]^+$.

*5.5. 2′-Hydroxy-3,4-dichlorochalcone **(5)***

Compound **1a** (5.5 g, 25 mmol), 3,4-di-chloro-benzaldehyde (4.38 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **5** (4.24 g, 14.5 mmol, 58%): IR (KBr) 3460, 1643, 1570 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 6.95 (1H, td, J = 8.1, 1.6 Hz, H-5′), 7.03 (1H, dd, J = 8.1, 1.6 Hz, H-3′), 7.43–7.56 (3H, m, H-4′, 5 and 6), 7.60 (1H, d, J = 15.5 Hz, H-α), 7.73 (1H, d, J = 1.7 Hz, H-2), 7.78 (1H, d, J = 15.5 Hz, H-β), 7.89 (1H, dd, J = 8.1, 1.6 Hz, H-6′), 12.66 (1H, s, OH-2′). ^{13}C-NMR ($CDCl_3$): δ 118.6 (C-2), 118.9 (C-3′), 119.7 (C-1′), 121.6 (C-5), 127.6 (C-α), 129.5 (C-6), 129.8 (C-5′), 130.9 (C-4′), 133.3 (C-1), 134.5 (C-3), 134.7 (C-4), 136.6 (C-6′), 142.4 (C-β), 163.6 (C-2′), 193.0 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 392 (22) $[M]^+$.

*5.6. 2′,5′-Dihydroxy-indol-3-yl-chalcone **(7)***

2′,5′-Bis(tetrahydropyran-2-yloxy)acetophenone (**7a**) was synthesis as previously reported [6]. Compound **7a** (8.00 g, 25 mmol), indole-3-aldehyde (3.62 g, 25 mmol) and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **7** (1.59 g, 5.73 mmol, 23%): IR (KBr) 3380, 1637, 1558 cm^{-1}. ^{1}H-NMR (acetone-d_6): δ 6.83 (1H, d, J = 8.8 Hz, H-3′), 7.09 (1H, dd, J = 8.8, 2.8 Hz, H-4′), 7.26–7.31 (2H, m, H-5, 6), 7.54–7.58 (1H, m, H-7), 7.60 (1H, d, J = 2.8 Hz, H-6′), 7.74 (1H, d, J = 15.2 Hz, H-α), 8.08 (1H, s, H-2), 8.10–8.14 (1H, m, H-4), 8.25 (1H, d, J = 15.2 Hz, H-β), 12.70 (1H, s, OH-2′). ^{13}C-NMR (acetone-d_6): δ 114.0 (C-7), 115.2 (C-3), 115.6 (C-6′), 115.8 (C-α), 120.1 (C-3′), 121.5 (C-1′), 121.9 (C-4), 123.1 (C-5), 124.6 (C-6), 125.6 (C-4′), 127.0 (C-3a), 134.8 (C-2), 139.6 (C-7a), 141.6 (C-β), 150.7 (C-2′), 158.4 (C-5′), 194.9 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 279 (6.8) $[M]^+$.

*5.7. 3,4-Dichloro-2′,5′-dimethoxychalcone **(8a)***

2,5-Dimethoxyacetophenone (4.50 g, 25 mmol), 3,4-dichlorobenzaldehyde (4.38 g, 25 mmol), and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **8a** (4.27 g, 12.6 mmol, 50.4%). Compound **8a** was identified with various spectral data compared with those of authentic sample [6].

*5.8. 3,4-Dichloro-2′,5′-dihydroxychalcone **(9a)***

2,5-Dihydroxyacetophenone (3.80 g, 25 mmol) was treated as in synthesis of 2,5-bis(tetrahydropyran-2-yloxy)-acetophenone (**7a**) [6] to give **7a**. Compound **7a** (8.0 g, 25 mmol), 3,4-dichlorobenzaldehyde (4.38 g, 25 mmol) and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **9a** (5.02 g, 16.3 mmol, 65%). The **9a** was identified with various spectral data and compared with those of authentic sample [6].

*5.9. 4-Chloro-2′,5′-dihydroxychalcone **(10a)***

2,5-Dihydroxyacetophenone (3.80 g, 25 mmol) was treated as in the synthesis of **9a** to give **7a**. Compound **7a** (8.0 g, 25 mmol), 4-chloro-benzylaldehyde (3.50 g,

25 mmol) and barium hydroxide octahydrate (4.29 g, 25 mmol) were treated as in the synthesis of **1** to give **10a**. The **10a** was identified with various spectral data and compared with those of authentic sample [4].

5.10. 3,5-Di-tert-butyl-2′,4,5′-trihydroxychalcone (6)

2,5-Dihydroxyacetophenone (3.80 g, 25 mmol) and 3,5-di-tert-butyl-4-hydroxybenzaldehyde (5.80 g, 25 mmol) dissolved in 50 ml of acetone. Al_2O_3 (12 g) was added to the above mixture of acetone solution at room temperature and stirred for 1 min, and the solvent was removed under reduced pressure. The reaction mixture was then subjected to sonic agitation (Prothermo Shaker NTS-211) at 40 °C for 12 h [28]. The product was eluted through a alumina column with *n*-hexane/CH_2Cl_2 (1:9) to give **6** (0.96 g, 2.61 mmol, 10.4%): IR (KBr) 3387, 1639, 1563 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 1.48 (18H, s, C(CH_3)$_3$ × 2), 5.63 (1H, s, OH-5′), 6.92 (1H, d, J = 8.7 Hz, H-3′), 7.04 (1H, dd, J = 8.7, 2.9 Hz, H-4′), 7.39 (1H, d, J = 2.9 Hz, H-6), 7.40 (1H, d, J = 15.3 Hz, H-α), 7.50 (2H, s, H-2 and 6), 7.91 (1H, d, J = 15.3 Hz, H-β), 12.55 (1H, s, OH-2′), ^{13}C-NMR ($CDCl_3$): δ 30.1 (–CH_3), 34.3 (–C(CH_3)$_3$), 114.6 (C-6′), 116.5 (C-3′), 119.2 (C-α), 119.9 (C-1′), 124.4 (C-4′), 125.9 (C-3 and 5), 126.3 (C-2 and 6), 136.6 (C-1), 147.3 (C-β), 147.4 (C-4), 157.0 (C-5′), 157.6 (C-2′), 193.3 (C=O). EIMS (70 eV) *m/z* (% rel. int.): 368 (31) $[M]^+$. HREIMS *m/z* $[M]^+$ 368.1988 (calcd. for $C_{23}H_{28}O_4$, 368.1987).

General Procedure for obtaining dihydrochalcones **8**–**11**.

5.11. 3,4-Dichloro-2′,5′-dimethoxydihydrochalcone (8)

A solution of **8a** (8.50 g, 25 mmol) in ethyl acetate (50 ml) was hydrogenated in an autoclave for 3 h at an initial pressure 60 kg cm^{-3} in the presence of 5% Pd/C (100 mg) at room temperature. The catalyst was removed by filtration and the filtrate was evaporated in vacuo and purified by chromatography over a silica gel column using a *n*-hexane/ethyl acetate (5:1) mixture as eluant to afford the crude product. The crude product was recrystallized from $CHCl_3$ to give **8** (4.27 g, 12.6 mmol, 50.4%): IR (KBr) 1662, 1596 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 2.97 (2H, t, J = 14.6 Hz, –$COCH_2CH_2$–), 3.29 (2H, t, J = 14.6 Hz, –$COCH_2CH_2$–), 3.78 (3H, s, OMe), 3.85 (3H, s, OMe), 6.89 (1H, d, J = 8.9 Hz, H-3′), 7.02 (1H, dd, J = 8.9, 3.0 Hz, H-4′), 7.09 (1H, d, J = 1.7 Hz, H-2), 7.25 (1H, d, J = 3.0 Hz, H-6′), 7.32 (1H, dd, J = 8.1, 1.7 Hz, H-6), 7.32 (1H, d, J = 8.1 Hz, H-5). ^{13}C-NMR ($CDCl_3$): δ 29.4 (–$COCH_2CH_2$–), 44.7 (–$COCH_2CH_2$–), 55.7 (OMe), 55.9 (OMe), 112.9 (C-6′), 113.8 (C-3′), 120.1 (C-4′), 127.8 (C-1′), 127.9 (C-5), 129.6 (C-1), 130.1 (C-2), 130.3 (C-6), 132.2 (C-4), 141.9 (C-3), 153.0 (C-5′), 153.3 (C-2′), 200.1 (CO). EIMS (70 eV) *m/z* (% rel. int.): 338 (9) $[M]^+$.

5.12. 3,4-Dichloro-2′,5′-dihydroxydihydrochalcone (9)

A solution of **9a** (7.70 g, 25 mmol) and 5% Pd/C (100 mg) in ethyl acetate were treated as in the synthesis of **8** to give **9** (3.30 g, 10.8 mmol, 43.2%): IR (KBr) 3335, 1642, 1514 cm^{-1}. ^{1}H-NMR (acetone-d_6): δ 3.04 (2H, t, J = 14.8 Hz, –$COCH_2CH_2$–), 3.44 (2H, t, J = 14.8 Hz, –$COCH_2CH_2$–), 6.81 (1H, d, J = 8.8 Hz, H-3), 7.09 (1H, dd, J = 8.8, 3.0 Hz, H-4′), 7.31 (1H, d, J = 8.3, 2.1 Hz, H-6), 7.34 (1H, t, J = 3.0 Hz, H-6′), 7.46 (1H, d, J = 8.3 Hz, H-5), 7.55 (1H, d, J = 2.1 Hz, H-2), 11.65 (1H, s, OH-2′). ^{13}C-NMR (acetone-d_6): δ 30.0 (–$COCH_2CH_2$–), 40.5 (–$COCH_2CH_2$–), 116.1 (C-6′), 120.1 (C-3′), 120.5 (C-1′), 126.3 (C-4′), 130.2 (C-6), 130.8 (C-3), 131.8 (C-5), 132.1 (C-2), 133.0 (C-4), 143.9 (C-1), 150.8 (C-5′), 157.1 (C-2′), 206.9 (CO). EIMS (70 eV) *m/z* (% rel. int.): 310 (12) $[M]^+$.

5.13. 5′-Allyloxy-4-chloro-2′-dihydroxydihydrochalcone (10)

A solution of **10a** (5.48 g, 20 mmol) and 5% Pd/C (100 mg) in ethyl acetate were treated as in the synthesis of **8** to give 4-chloro-2′,5′-dihydroxydihydrochalcone (**10b**) (2.76 g, 10 mmol). Compound **10b** was identified with various spectral data and compared with those of authentic sample [4]. 3-Bromo-1-propene (1.20 g, 10 mmol) was added to a solution of **10b** (2.76 g, 10 mmol) and anhydrous K_2CO_3 (6.00 g, 10 mmol) in DMF (50 ml) and stirred at room temperature for 18 h. The mixture was diluted with water and washed three times with water. The organic phase was dried over sodium sulfate, filtered and concentrated in vacuo to give the product. The product was purified by chromatography over a silica gel column using a *n*-hexane/ethyl acetate (2:1) mixture as eluant to afford **10** (0.35 g, 1.12 mmol, 11.2%): IR (KBr) 3387, 1646, 1617 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 3.03 (2H, t, J = 14.6 Hz, –$COCH_2CH_2$–), 3.27 (2H, t, J = 14.6 Hz, –$COCH_2CH_2$–), 4.48 (2H, d, J = 5.2 Hz, CH_2=CHCH_2–), 5.30 (1H, d, J_E = 11.8 Hz, H_2C=$CHCH_2$–), 5.40 (1H, d, J_Z = 17.2 Hz, H_2C=$CHCH_2$–), 5.94–6.13 (1H, m, H_2C=CH–CH_2–), 6.90 (1H, d, J = 8.9 Hz, H-3′), 7.31–7.09 (6H, m, H-2, 3, 4′, 5, 6, 6′), 11.84 (1H, s, OH-2′). ^{13}C-NMR ($CDCl_3$): δ 29.2 (–$COCH_2CH_2$–), 39.7 (–$COCH_2CH_2$–), 69.8 (–OCH_2–), 114.0 (H_2C=$CHCH_2$–), 117.9 (C-3′), 118.7 (C-1′), 119.3 (C-6′), 124.9 (C-4′), 128.6 (C-3 and 5), 129.7 (C-2 and 6), 132.0 (C-4), 133.0 (H_2C=$CHCH_2$–), 139.1 (C-1), 150.6 (C-5′), 156.9 (C-2′), 204.3 (CO). EIMS (70 eV) *m/z* (% rel. int.): 316 (1) $[M]^+$.

5.14. 4-Chloro-2′,5′-dihydroxy-3′-(1-methylallyl)dihydrochalcone (11)

3-Bromo-1-butene (1.35 g, 10 mmol) was added to a solution of **10b** (2.76 g, 10 mmol) and anhydrous K_2CO_3 (6.00 g, 10 mmol) in DMF (50 ml) and refluxed for 18 h. The mixture was treated as in the synthesis of **10** to give **11** (0.207 g, 0.63 mmol, 7.3%): IR (KBr) 3421, 1637, 1591 cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 1.29 (3H, d, J = 7.0 Hz, CH_3), 2.99 (2H, t, J = 14.0 Hz, –$COCH_2CH_2$–), 3.20 (2H, t, J = 14.0 Hz, –$COCH_2CH_2$–), 3.95 (1H, m, –CH(CH_3)–), 5.06 (1H, m, J_E = 10.3 Hz, H_2C=CH–), 5.08 (1H, m, J_Z = 17.2 Hz,

H_2C=CH–), 5.70 (1H, s, OH-5′), 5.90–6.07 (1H, m, H_2C=C*H*–), 6.95 (1H, d, *J* = 2.9 Hz, H-6′), 7.05 (1H, d, *J* = 2.9 Hz, H-4′), 7.13 (2H, dd, *J* = 8.2, 2.1 Hz, H-3 and 5), 7.24 (2H, dd, *J* = 8.2, 2.1 Hz, H-2 and 6), 12.3 (1H, s, OH-2′). ^{13}C-NMR ($CDCl_3$): δ 18.8 (CH_3), 29.2 (–CO–CH_2*C*H_2–), 34.9 (=CH–*C*H(CH_3)–), 39.8 (–CO*C*H_2–), 112.2 (C-6′), 113.7 (H_2*C*=CH–), 118.4 (C-1′), 123.0 (C-4′), 128.6 (C-3 and 5), 129.7 (C-2 and 6), 132.0 (C-3′), 136.3 (C-1), 139.0 (C-4), 141.5 (H_2C=*C*H–), 147.0 (C-5′), 154.3 (C-2′), 204.7 (CO). EIMS (70 eV) *m/z* (% rel. int.): 330 (29) $[M]^+$.

5.15. Tumor cell growth inhibition assays

A microassay for cytotoxicity was performed using MTT [36,37]. Briefly, 1–3 × 10^3 cells/100 μl were seeded in 96-well microplates (Nunk, Roskidle, Denmark) and preincubated for 6 h to allow attachment. The cells were incubated with each drug for 6 days and then pulsed 10 μl of MTT (5 mg ml^{-1}; Sigma, St. Louis, MO) and incubated for an additional 4 h at 37 °C. The microplates were read 550 nm on a *Multiskan Photometer* (MRX II; Dynatech, McLean, VA) after lysis of cells with 100 μl of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl. Control wells contained medium and cells (total absorbance) or medium alone (background absorbance). Cell death was calculated as the percentage of MTT inhibition.

Human hepatomacellular carcinoma Hep 3B, T-24, human colorectal adenocarcinoma HT-29, and human breast adenocarcinoma MCF-7 cells were obtained from American Type Culture Collection (ATCC; Rockville, MO) and grown in DMEM [37,38], containing 10% FBS, 2 mM L-glutamine, 100 units ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin. For the microassay, the growth medium was supplemented with 10 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer pH 7.3 and incubated at 37 °C in a CO_2 incubator.

5.16. Flow cytometry

Compounds were added to cells (1 × 10^7 cell ml^{-1}), respectively. At various time intervals, the reactions were terminated by washing with PBS. The cells were fixed with 4% paraformaldehyde/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 μg ml^{-1}) 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.

5.17. DNA fragmentation assay

After tested drug treatment for various time intervals, cells in 150-mm plates were harvested and washed with PBS. After the addition of 100 μl lysis buffer [1% of NP-40 (Sigma) in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5] and mixing; the cell lysates were then centrifuged and the supernatants were collected. The supernatants were incubated with 50 μl of RNase A (20 mg ml^{-1}) and 20 μl of SDS (10%) at 56 °C for 2 h. Then, 35 μl of proteinase K (20 mg ml^{-1}) was added and incubated at 37 °C overnight. DNA fragments were precipitated after the addition of 150 μl of 10 M NH_4OAc and 1.2 ml of 100% ethanol at −20 °C overnight. After centrifuging and drying, the DNA pellets were suspended in 15 μl Tris–EDTA buffer and electrophoresed on a 1% (W/V) agarose gel in TBE buffer at 50 V for 1 h, DNA laddering was observed after staining with ethidium bromide solution and exposing to the UV light [39].

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