

## Full Paper

# Synthesis and Anti-Cancer Activity Evaluation of New Dimethoxylated Chalcone and Flavanone Analogs

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A novel series of chalcones and flavanones discriminated by the presence of a 3,4-dimethoxyphenyl moiety in their structures were synthesized as anti-cancer agents. The cytotoxicity evaluation of the analogs against the MCF-7, MDA-MB-231 (human breast cancer), and SK-N-MC (human neuroblastoma) cell lines demonstrated that the introduction of a halogen on the 3,4-dimethoxyphenyl part of both series and the attachment of a pyrrolidinylethoxy group on the C-7 position of the flavanone derivatives increased their activity. Indeed, 3-halogenated chalcones (**1c** and **1d**) were more potent than the standard drug etoposide against all tested cell lines. Fluorescence microscopy and flow cytometry analyses confirmed that the anti-cancer effect of the most potent compounds **1c** and **1d** occurs via apoptosis induction.

**Keywords:** Apoptosis / Anti-cancer agents / Chalcone / Cytotoxicity / Flavanone

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## Introduction

Cancer is characterized by uncontrolled growth of abnormal cells in the body [1]. It is a serious cause of mortality in the present society [2]. Although there are outstanding developments in diagnostic methods and therapeutic approaches, cancer is one of the most frequent clinical problems worldwide [3]. Nowadays, chemotherapy is one of the notable healing approaches. Since most of the anti-cancer agents have toxic effects on the normal cells, chemotherapy leads to various adverse reactions. Therefore, designing safe and effective new anti-cancer drugs is a necessary issue in the medicinal chemistry [4].

Plants are remarkable source of anticancer drugs and lead compounds. Among natural products, flavonoids are one of the most important groups [5]. More than 4000 flavonoids have been found in plants [6]. Dietary flavonoids usually exist in nuts, fruits, vegetables, spices, and seeds [5, 6] and exhibit wide spectrum of biological activities such as anti-inflammatory, antioxidant, and antiproliferation [7–9].

Structure of flavonoids includes phenyl benzopyrone (C6-C3-C6 skeleton) or 1,3-diarylpropane skeleton in which arrangement of skeleton depends on degree of oxidation [7, 10]. This large family is classified into flavons, flavonols, flavanones, isoflavones, flavanes, chalcones, and anthocyanidins [11, 12]. Chalcones are bicyclic flavonoids that consist of two aromatic rings that are linked by an  $\alpha,\beta$ -unsaturated ketone system [13]. They display several pharmacological activities such as antibacterial, antifungal, antileishmanial, and antitumor [14–17]. Recently, many derivatives of chalcones were synthesized for evaluation of their cytotoxic activity. According to the results of various studies, chalcones,

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dihydrochalcones, bis-chalcones, or hybrids of chalcone with coumarin exhibited anti-proliferative activity against different cancer cell lines especially breast and prostate cancers [1, 5, 18–20]. Rao *et al.* [2] demonstrated that chalcones with methoxy groups on aromatic rings have potent anti-proliferative effects on tumor cell line without cytotoxicity on normal cells.

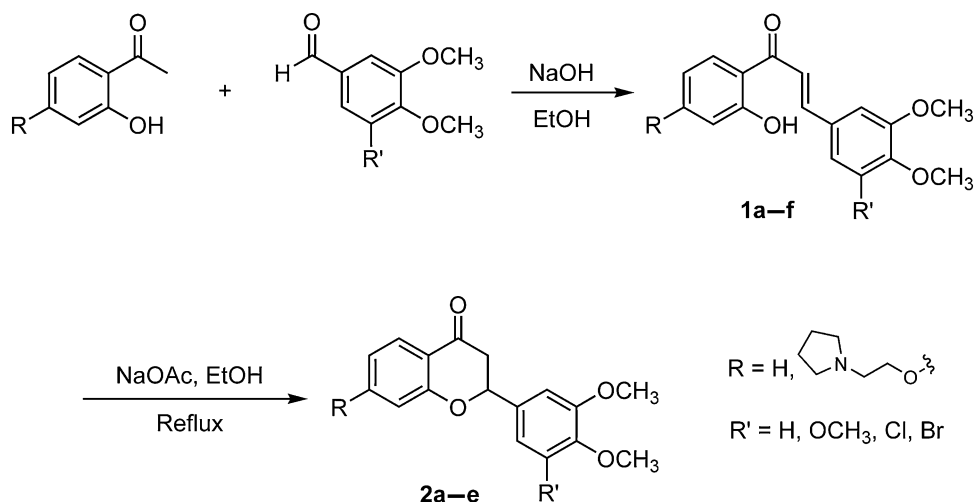
Besides chalcones, current reports are focused on anti-tumor activity of flavanones. Min *et al.* synthesized flavanone analogs with cytotoxic activity on leukemia and lung carcinoma cells [21]. Derivatives consist of methoxy and halogen groups on aromatic rings exhibiting inhibitory effect on proliferation of human breast cancer cell lines [8, 22]. Furthermore, it was illustrated that the halogenated flavanones can induce apoptosis [7, 8].

According to the available data, we designed and synthesized a novel series of chalcones and flavanones discriminated by presence of 3,4-dimethoxyphenyl moiety in their structures to find new potential anti-cancer agents. The cytotoxicity of compounds was determined by MTT assay. Finally, assessment of apoptosis induction of more potent compounds was performed.

## Results and discussion

### Chemistry

Chalcones **1a–f** were obtained from Claisen–Schmidt condensation of an appropriate acetophenone with various benzaldehydes in the presence of sodium hydroxide. The second series of compounds (flavanones) **2a–e** were prepared by cyclizing of chalcone derivatives **1** in refluxing ethanol and in the presence of sodium acetate (Scheme 1) [7].



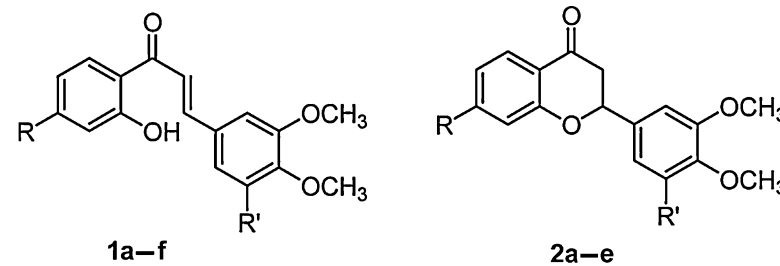
**Scheme 1.** Synthesis of chalcone and flavanone derivatives.

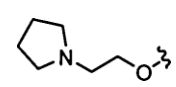
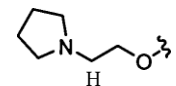
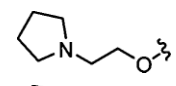
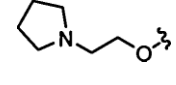
The structures of compounds were confirmed by FT-IR and <sup>1</sup>H NMR spectroscopy. In the <sup>1</sup>H NMR spectra of chalcones, the signal of phenolic hydroxyl group appeared at 12.77–13.53 ppm. Also, the protons of olefinic group resonanced at 7.81–7.94 and 7.46–7.59 ppm as two doublets. Coupling constant for olefinic protons was about 15.2–16.0 Hz in accord to *trans*-configuration for chalcone derivatives. In the IR spectra, the carbonyl stretching vibration band of chalcones **1a–f** appeared at 1631–1687 cm<sup>-1</sup>. The observed band at 3420–3443 cm<sup>-1</sup> was related to the phenolic OH of chalcones **1a–f**. In the <sup>1</sup>H NMR of flavanones **2a–e**, there were three peaks approximately at 5.4, 3.1, and 2.9 ppm. The pattern of these peaks was characteristic of protons located at the C2 and C3 position of chroman ring [7]. The band due to the C=O stretching of flavanones **2a–e** occurred at 1673–1696 cm<sup>-1</sup>.

### Biology

The cytotoxic activity of synthesized compounds was evaluated against three human cancer cell lines, including MCF-7, MDA-MB-231, and SK-N-MC by MTT assay. The inhibition percentages of compounds were assessed by the formula [(absorbance of control cells – absorbance of treated cells)/absorbance of control cells] × 100. The concentration causing 50% cell growth inhibition (IC<sub>50</sub>) was calculated from concentration–response curves by regression analysis.

Table 1 shows the IC<sub>50</sub> value of synthesized compounds against three human cancer cell lines. All compounds with the exception of **1e** and **2b** showed good activity against SK-N-MC cells (IC<sub>50</sub> values ≤ 16.8 μM). Compounds **1a–d** and **2c** were more potent than standard drug etoposide against these cells. Also, the cytotoxic activity of compounds **2d** and **2e** against SK-N-MC was equal to that of etoposide. In the case of

**Table 1.** Results of MTT assay ( $IC_{50}$ ,  $\mu M$ ) of compounds **1a–f** and **2a–e** against three human cancer cell lines.


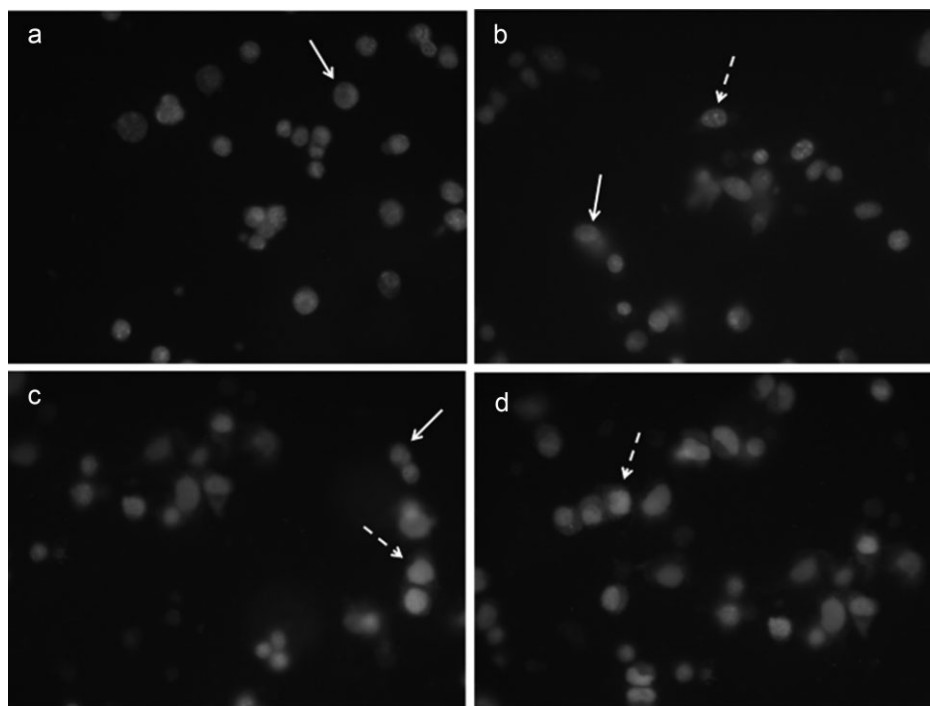
Compounds	R	R'	SK-N-MC	MDA-MB-231	MCF-7
<b>1a</b>	H	H	2.5 ± 0.8	6.9 ± 2.6	6.1 ± 2.1
<b>1b</b>	H	OCH <sub>3</sub>	4.7 ± 2.3	9.8 ± 1.3	6.5 ± 0.7
<b>1c</b>	H	Cl	1.5 ± 0.2	6.2 ± 0.8	1.2 ± 0.6
<b>1d</b>	H	Br	1.1 ± 0.6	1.3 ± 0.3	1.0 ± 0.6
<b>1e</b>		H	57.7 ± 4.5	>100	>100
<b>1f</b>		OCH <sub>3</sub>	11.9 ± 1.4	>100	14.0 ± 1.3
<b>2a</b>	H	H	16.8 ± 7.2	16.3 ± 2.9	10.6 ± 6.4
<b>2b</b>	H	OCH <sub>3</sub>	39.3 ± 7.4	>100	14.5 ± 0.7
<b>2c</b>	H	Cl	3.7 ± 0.8	11.5 ± 1.8	9.7 ± 3.9
<b>2d</b>		H	7.9 ± 1.7	13.9 ± 3.2	8.9 ± 1.9
<b>2e</b>		OCH <sub>3</sub>	7.9 ± 3.7	11.8 ± 5.9	13.1 ± 6.5
Etoposide			8.3 ± 3.9	7.1 ± 2.6	8.9 ± 2.4

breast cancer cells (MDA-MB-231 and MCF-7), most compounds displayed significant activity with  $IC_{50}$  values of 1.0–16.3  $\mu M$ . In general, bromochalcone **1d** was the most potent compound against all tested cell lines. It was five to eight times more potent than etoposide.

The tested compounds were structurally related to chalcones and flavanones. The comparison of chalcone derivatives with flavanone analogs indicated that chalcones (**1a–c**) were more potent than corresponding flavanone (**2a–c**) against all tested cancer cell lines. In contrast, flavanone compounds containing pyrrolidinylethoxy moiety (**2d** and **2e**) are more potent than related chalcones analogs (**1e** and **1f**). The observed  $IC_{50}$  values revealed that the insertion of chlorine or bromine atom on 3,4-dimethoxyphenyl part of the molecules (both chalcones and flavanones) improved the profile of cytotoxicity. However, the introduction of third methoxy substituent on the 3,4-dimethoxyphenyl moiety could not improve the activity and even in some cases diminished the cytotoxicity. Also, pyrrolidinylethoxy substituent on C-7 position of flavanone derivatives was favorable for anti-proliferative effect of these compounds.

The cytotoxic activity of chlorochalcone **1c** and bromochalcone **1d** as the most potent compounds was evaluated on non-tumoral cell line MRC-5. According to the results, the  $IC_{50}$  values of compounds **1c** and **1d** on human normal cell line were >100  $\mu M$  and  $31 \pm 3.1 \mu M$ , respectively. Therefore, the cytotoxicity of these compounds was apparently selective for cancer cells, and compounds **1c** and **1d** had less cytotoxic activity toward normal human cell line MRC-5.

Apoptosis induction by the selected compounds **1c** and **1d** was evaluated using acridine orange/ethidium bromide double staining technique. Analysis of the acridine orange/ethidium bromide staining showed that the compounds **1c** and **1d** induced apoptosis in MCF-7 cell line. As shown in Fig. 1, the viable cells were observed green and the apoptotic cells had orange particles in their nuclei. These results were also established by the flow cytometry analyses of the apoptotic cells. We used annexin V/7-AAD flow cytometric analysis as a quantitative method of determining apoptosis. According to the results, the cells undergo apoptosis after treatment with  $IC_{50}$  concentrations of **1c** and **1d**. As shown in Fig. 2, compounds **1c** and **1d** induced 17.9 and 17% apoptosis in the MCF-7 cells, respectively. These percentages are



**Figure 1.** Acridine orange/ethidium bromide double staining of MCF-7 cells with characteristic symptoms of apoptosis: (a) DMSO 1% as control, (b) etoposide as positive control, (c and d) cells treated with  $IC_{50}$  of compounds **1c** and **1d**, respectively, for 24 h. White arrow indicates live cells and dashed arrow indicates apoptotic cells. The images of cells were taken with a fluorescence microscope at  $400\times$ .

implicated on the cells in the early stages of apoptosis. Therefore, the evaluation of apoptosis in cells treated with the test compounds confirmed the anti-proliferative activity. These data confirmed compounds **1c** and **1d** induce apoptosis in MCF-7 cell line. Also percentages of apoptosis for compounds **1c** and **1d** are comparable with etoposide as reference drug, which induced 18.24% apoptosis in MCF-7 cells.

## Conclusion

In summary, to find new potential anti-cancer agents, we synthesized a novel series of chalcones and related flavanones discriminated by the presence of 3,4-dimethoxyphenyl moiety in their structures. The limited structure–activity relationships study demonstrated that the introduction of halogen on the 3,4-dimethoxyphenyl part of both series, and attachment of pyrrolidinyloxy group on the C-7 position of flavanone derivatives increased cytotoxic activity. Cytotoxicity evaluation revealed that 3'-halogenated chalcones (**1c** and **1d**) were more potent than etoposide and 7-(pyrrolylethoxy)flavanones **2e** and **2d** were as potent as etoposide. Moreover, cytotoxic activity of compounds **1c** and **1d** on human normal cell line (MRC-5) was significantly less than cancer cell lines. Thus, the anti-proliferative effect of these analogs was selective.

Apoptosis induction of compound **1c** and **1d** was assessed on MCF-7 cell line. According to acridine orange/ethidium bromide staining and flow-cytometric data, compounds **1c** and **1d** induce apoptosis in MCF-7 cell line.

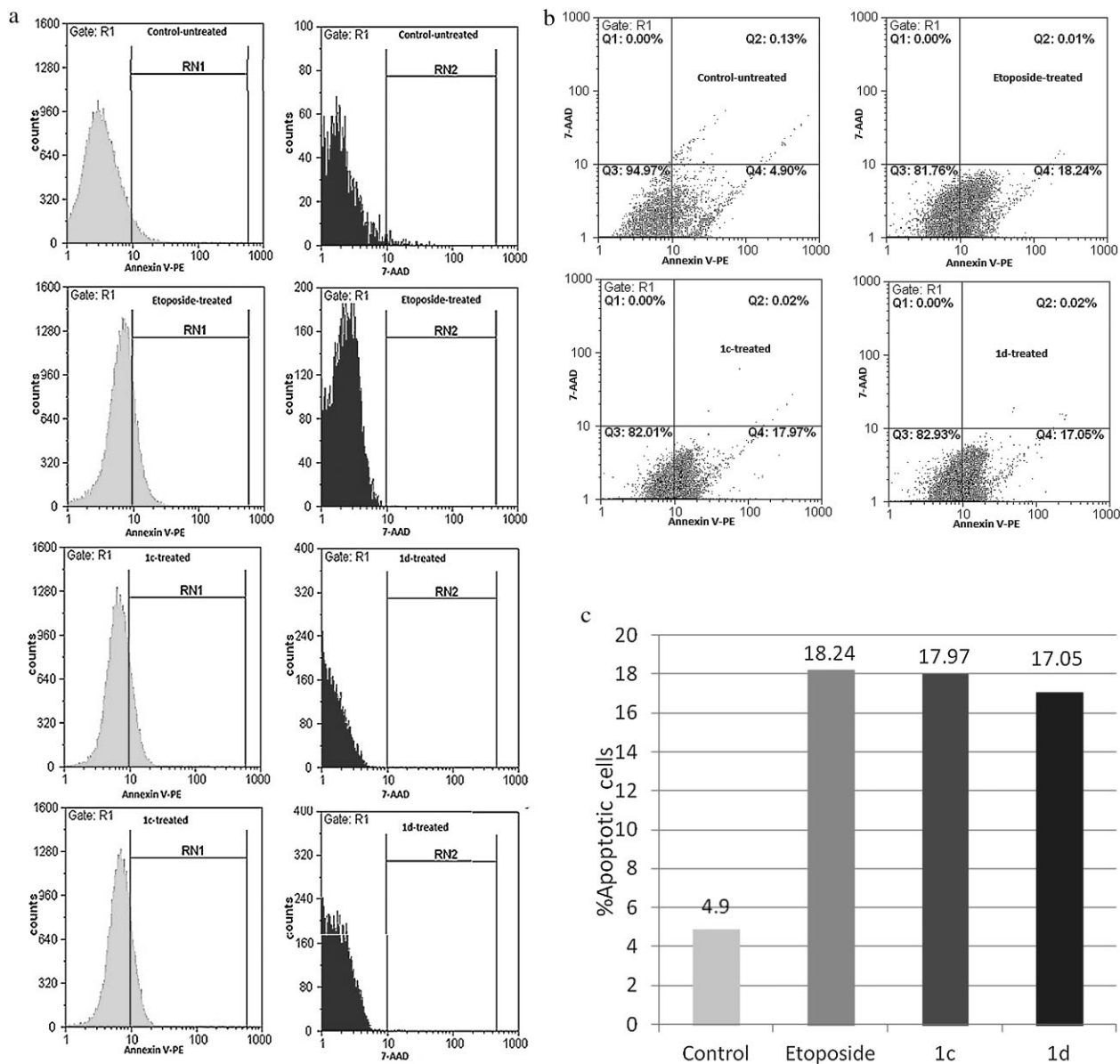
## Experimental

### Chemistry

Chemicals and all solvents used in this study were purchased from Merck AG and Aldrich Chemicals. Melting points were determined on a Kofler hot stage apparatus. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks).  $^1H$  NMR spectra were recorded using Bruker 400 spectrometer and chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard. Elemental analyses were carried out on a Costec rapid elemental analyzer model 4010 (Costec, Germany) for C and H, and the results are within  $\pm 0.4\%$  of the theoretical values. The chalcones **1a–f** and flavanone derivatives **2a–e** were synthesized starting from appropriate 2-hydroxyacetophenone and dimethoxybenzaldehyde as outlined in Scheme 1. 3-Chloro-4,5-dimethoxybenzaldehyde, 3-bromo-4,5-dimethoxybenzaldehyde, and 1-(2-hydroxy-4-(2-tetrahydro-1H-1-pyrrolylethoxy)phenyl)-1-ethanone were prepared according to the literature methods [23, 24].

### General procedure for the synthesis of chalcones **1a–f**

To a solution of substituted acetophenone (1 mmol) and appropriate 3,4-dimethoxybenzaldehyde (1 mmol) in ethanol



**Figure 2.** Apoptosis analysis of MCF-7 cells treated with compounds **1c** and **1d** by annexin V-PE/7-AAD double staining. (a) MCF-7 cells treated with DMSO 1% (negative control) or with  $IC_{50}$  values of etoposide (positive control) and compounds **1c** and **1d**. Fluorescence intensity of annexin V-PE was shifted to the right in cells treated with compounds **1c** and **1d**. (b and c) Percentage apoptotic cells (%PE-positive cells) was calculated. Exposure of MCF-7 cells to  $IC_{50}$  concentrations of etoposide, **1c** and **1d** induced apoptosis in 18.24, 17.97, and 17.05% of the cells, respectively.

(20 mL), 50% NaOH solution (4 mL) was added. Color of mixture changed and precipitate formed. Then, the volume of solution was increased up to 100 mL and it was stirred at room temperature over night. The precipitate was filtered and suspended in water. The suspension was acidified by 37% HCl and pH of solution was 3. The product was extracted with ethyl acetate three times and the organic layer was washed with water and brine, and then dried ( $Na_2SO_4$ ). After evaporation of solvent,

the residue was crystallized from ethanol or methanol to give compounds **1a–f**.

### 3-(3,4-Dimethoxyphenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**1a**)

Yield 90%; m.p. 100–102°C;  $^1H$  NMR ( $CDCl_3$ )  $\delta$ : 12.98 (s, 1H, OH), 7.94 (d, 1H hydroxyphenyl,  $J = 7.6$  Hz), 7.9 (d, 1H, vinyl,  $J = 15.2$

Hz), 7.53 (m, 2H, vinyl and hydroxyphenyl), 7.27 (m, 1H hydroxyphenyl), 7.17 (s, 1H phenyl), 7.03 (d, 1H phenyl,  $J = 8.8$  Hz), 6.96 (m, 2H, phenyl), 3.96 (s, 3H, OCH<sub>3</sub>), 3.94 (s, 3H, OCH<sub>3</sub>); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 3443 (OH), 1631 (C=O). Anal. calcd. for C<sub>17</sub>H<sub>16</sub>O<sub>4</sub>: C, 71.62; H, 5.67. Found: C, 71.51; H, 5.72.

### 1-(2-Hydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**1b**)

Yield 82%; m.p. 132–133°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 12.82 (s, 1H, OH), 7.95 (d, 1H hydroxyphenyl,  $J = 8.0$  Hz), 7.88 (d, 1H, vinyl,  $J = 16.0$  Hz), 7.56 (m, 2H, vinyl and hydroxyphenyl), 7.06 (d, 1H hydroxyphenyl,  $J = 8.0$  Hz), 6.89 (s, 1H phenyl), 6.16 (s, 1H phenyl), 3.94 (s, 3H, OCH<sub>3</sub>), 3.93 (s, 6H, OCH<sub>3</sub>); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 3438 (OH), 1637 (C=O). Anal. calcd. for C<sub>18</sub>H<sub>18</sub>O<sub>5</sub>: C, 68.78; H, 5.77. Found: C, 68.93; H, 5.94.

### 3-(3-Chloro-4,5-dimethoxyphenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**1c**)

Yield 36%; m.p. 113–116°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 12.77 (s, 1H, OH), 7.94 (d, 1H, hydroxyphenyl,  $J = 8.0$  Hz), 7.81 (d, 1H, vinyl,  $J = 13.6$  Hz), 7.59 (m, 2H, vinyl and hydroxyphenyl), 7.35 (s, 1H, phenyl), 7.06 (m, 3H, hydroxyphenyl and phenyl), 3.95 (s, 3H, OCH<sub>3</sub>), 3.93 (s, 3H, OCH<sub>3</sub>); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 3429 (OH), 1687 (C=O). Anal. calcd. for C<sub>17</sub>H<sub>15</sub>ClO<sub>4</sub>: C, 64.06; H, 4.74. Found: C, 63.94; H, 4.87.

### 3-(3-Bromo-4,5-dimethoxyphenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**1d**)

Yield 20%; m.p. 102–104°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 12.83 (s, 1H, OH), 7.94 (d, 1H, vinyl,  $J = 15.6$  Hz), 7.57 (m, 2H, vinyl and hydroxyphenyl), 7.09 (s, 1H phenyl), 7.05 (d, 1H phenyl,  $J = 8.0$  Hz), 6.97 (m, 2H, phenyl and hydroxyphenyl), 3.95 (s, 3H, OCH<sub>3</sub>), 3.92 (s, 3H, OCH<sub>3</sub>); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 3431 (OH), 1639 (C=O). Anal. calcd. for C<sub>17</sub>H<sub>15</sub>BrO<sub>4</sub>: C, 56.22; H, 4.16. Found: C, 56.35; H, 4.08.

### 3-(3,4-Dimethoxyphenyl)-1-(2-hydroxy-4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)prop-2-en-1-one (**1e**)

Yield 54%; m.p. 141–145°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 13.53 (s, 1H, OH), 7.88 (m, 2H, hydroxyphenyl and vinyl), 7.46 (d, 1H, vinyl,  $J = 15.2$  Hz), 7.17 (s, 1H, phenyl), 6.93 (d, 1H, phenyl,  $J = 8.0$  Hz), 6.54 (m, 3H, phenyl and hydroxyphenyl), 4.29 (bs, 2H, alkane), 3.97 (s, 3H, OCH<sub>3</sub>), 3.95 (s, 3H, OCH<sub>3</sub>), 3.85 (bs, 2H, alkane), 1.65 (m, 8H, pyrrolidine); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 3442 (OH), 1630 (C=O). Anal. calcd. for C<sub>23</sub>H<sub>27</sub>NO<sub>5</sub>: C, 69.5; H, 6.85. Found: C, 69.27; H, 6.7.

### 1-(2-Hydroxy-4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**1f**)

Yield 41%; m.p. 120–123°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 13.42 (s, 1H, OH), 7.87 (m, 2H, vinyl and hydroxyphenyl), 7.47 (d, 1H, vinyl,  $J = 15.6$  Hz), 6.87 (s, 2H, phenyl), 6.54 (m, 2H, hydroxyphenyl), 4.26 (m, 2H, alkane), 3.94 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 6H, OCH<sub>3</sub>), 3.87 (m, 2H, alkane), 1.65 (bs, 4H, pyrrolidine); 1.47 (m, 4H, pyrrolidine); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 3420 (OH), 1638 (C=O). Anal. calcd. for C<sub>24</sub>H<sub>29</sub>NO<sub>6</sub>: C, 67.3; H, 6.84. Found: C, 67.52; H, 6.79.

### General procedure for the synthesis of flavanones **2a–e**

A solution of an appropriate chalcone (1 mmol) in ethanol (10 mL) was treated with sodium acetate (600 mg) and two drops of water.

The mixture was refluxed for 48 h. It was poured in cold water and the precipitate was extracted with ethyl acetate. Organic phase was dried (Na<sub>2</sub>SO<sub>4</sub>) and the solvent was removed. The residue was crystallized from methanol or ethanol to give corresponding flavanone.

### 2-(3,4-Dimethoxyphenyl)-2,3-dihydrochromen-4-one (**2a**)

Yield 57%; m.p. 118–120°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 7.95 (dd, 1H,  $J = 8.0$  and 1.6 Hz, H<sub>5</sub> chroman), 7.51 (td, 1H,  $J = 8.0$  and 2.0 Hz, H<sub>7</sub> chroman), 7.07 (m, 4H, H<sub>6</sub>, H<sub>8</sub> chroman and H<sub>2'</sub>, H<sub>6'</sub>), 6.95 (d, 1H,  $J = 8.0$  Hz, H<sub>5'</sub>), 5.45 (dd, 1H,  $J = 15.2$  and 2.8 Hz, H<sub>2</sub> chroman), 3.93 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 3.16 (t, 1H,  $J = 15.2$  Hz, H<sub>3</sub> chroman), 2.9 (dd, 1H,  $J = 15.2$  and 2.8 Hz, H<sub>3</sub> chroman); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 1696 (C=O). Anal. calcd. for C<sub>17</sub>H<sub>16</sub>O<sub>4</sub>: C, 71.62; H, 5.67. Found: C, 71.87; H, 5.97.

### 2-(3,4,5-Trimethoxyphenyl)-2,3-dihydrochromen-4-one (**2b**)

Yield 54%; m.p. 123–127°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 7.95 (d, 1H,  $J = 7.2$  Hz, H<sub>5</sub> chroman), 7.53 (m, 2H, H<sub>6</sub> and H<sub>7</sub> chroman), 7.35 (s, 1H, H<sub>2'</sub>), 7.09 (d, 1H,  $J = 7.2$  Hz, H<sub>8</sub> chroman), 6.71 (s, 1H, H<sub>6'</sub>), 5.43 (d, 1H,  $J = 15.6$  Hz, H<sub>2</sub> chroman), 3.9 (s, 6H, OCH<sub>3</sub>), 3.14 (t, 1H,  $J = 15.6$  Hz, H<sub>3</sub> chroman), 2.91 (d, 1H,  $J = 15.6$  Hz, H<sub>3</sub> chroman); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 1688 (C=O). Anal. calcd. for C<sub>18</sub>H<sub>18</sub>O<sub>5</sub>: C, 68.78; H, 5.77. Found: C, 68.88; H, 5.54.

### 2-(3-Chloro-4,5-dimethoxyphenyl)-2,3-dihydrochromen-4-one (**2c**)

Yield 10%; m.p. 120–123°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 7.94 (d, 1H,  $J = 7.6$  Hz, H<sub>5</sub> chroman), 7.55 (t, 1H,  $J = 7.2$  Hz, H<sub>7</sub> chroman), 7.11 (m, 3H, H<sub>6</sub> and H<sub>8</sub> chroman and H<sub>2'</sub>), 6.97 (s, 1H, H<sub>6'</sub>), 5.41 (d, 1H,  $J = 13.2$  Hz, H<sub>2</sub> chroman), 3.91 (s, 3H, OCH<sub>3</sub>), 3.88 (s, 3H, OCH<sub>3</sub>), 3.09 (t, 1H,  $J = 13.2$  Hz, H<sub>3</sub> chroman), 2.91 (d, 1H,  $J = 13.2$  Hz, H<sub>3</sub> chroman); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 1687 (C=O). Anal. calcd. for C<sub>17</sub>H<sub>15</sub>ClO<sub>4</sub>: C, 64.06; H, 4.74. Found: C, 63.83; H, 4.64.

### 2-(3,4-Dimethoxyphenyl)-7-(2-(pyrrolidin-1-yl)ethoxy)-2,3-dihydrochromen-4-one (**2d**)

Yield 23%; m.p. 132–135°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 7.9 (d, 1H,  $J = 8.4$  Hz, H<sub>5</sub> chroman), 7.01 (m, 2H, H<sub>2'</sub> and H<sub>6'</sub>), 6.92 (d, 1H,  $J = 8.0$  Hz, H<sub>5'</sub>), 6.65 (d, 1H,  $J = 8.4$  Hz, H<sub>6</sub> chroman), 6.5 (s, 1H, H<sub>8</sub> chroman), 5.43 (d, 1H,  $J = 15.0$  Hz, H<sub>2</sub> chroman), 4.25 (bs, 2H, alkane), 3.93 (s, 3H, OCH<sub>3</sub>), 3.91 (s, 3H, OCH<sub>3</sub>), 3.82 (bs, 2H, alkane), 3.11 (t, 1H,  $J = 15.0$  Hz, H<sub>3</sub> chroman), 2.85 (d, 1H,  $J = 15.0$  Hz, H<sub>3</sub> chroman), 1.55 (m, 8H, pyrrolidine); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 1673 (C=O). Anal. calcd. for C<sub>23</sub>H<sub>27</sub>NO<sub>5</sub>: C, 69.5; H, 6.85. Found: C, 69.78; H, 6.98.

### 7-(2-(Pyrrolidin-1-yl)ethoxy)-2-(3,4,5-trimethoxyphenyl)-2,3-dihydrochromen-4-one (**2e**)

Yield 20%; m.p. 75–77°C; <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 7.9 (d, 1H,  $J = 8.8$  Hz, H<sub>5</sub> chroman), 6.69 (m, 3H, H<sub>2'</sub> and H<sub>6'</sub> and H<sub>6</sub> chroman), 5.41 (d, 1H,  $J = 14.5$  Hz, H<sub>2</sub> chroman), 4.27 (m, 2H, alkane), 3.9 (s, 3H, OCH<sub>3</sub>), 3.87 (s, 6H, OCH<sub>3</sub>), 3.04 (t, 1H,  $J = 14.5$  Hz, H<sub>3</sub> chroman), 2.86 (d, 1H,  $J = 14.5$  Hz, H<sub>3</sub> chroman), 1.54 (bs, 4H, pyrrolidine), 1.27 (bs, 4H, pyrrolidine); IR (KBr, cm<sup>-1</sup>)  $\nu_{\max}$ : 1679 (C=O). Anal. calcd. for C<sub>24</sub>H<sub>29</sub>NO<sub>6</sub>: C, 67.3; H, 6.84. Found: C, 67.54; H, 6.98.

## Biology

### Cell lines and cell culture

Human cancer cell lines including MCF-7, MDA-MB-231, and SK-N-MC were purchased from Pasteur Institute of Iran. The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (from Gibco BRL, UK) and 100 µg/mL streptomycin and 100 U/mL penicillin at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air.

### MTT assay

The cytotoxicity evaluation of compounds **1a–f** and **2a–e** on three human cancer cell lines including MCF-7 and MDA-MB-231 (human breast cancer cells), and SK-N-MC (human neuroblastoma) was performed by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay [25]. Also, the cytotoxic activity of compounds **1c** and **1d** were determined on MRC-5 (human normal lung cell line).

Briefly, cells in the log-phase of growth were harvested by trypsinization, seeded in 96-well plates (Nunc, Denmark) for 24 h. Then, the cells were treated with various final concentrations (1, 5, 10, and 20 µg/mL) of the compounds for 48 h. Etoposide and DMSO were used as positive and negative controls, respectively. The highest concentration of DMSO in the final step was 1%. After 48 h, the culture medium was removed, and cells were incubated with 200 µL of 0.5 mg/mL solution (final concentration) of MTT (Sigma–Aldrich) for 4 h. Then, the supernatant was removed and the formazan crystals were dissolved using DMSO. The absorbance was read at 492 nm with an ELISA plate reader (Biotek Instruments, Winooski, Vt.) after 30 min.

### Acridine orange/ethidium bromide staining method

Apoptosis was determined morphologically by staining with acridine orange/ethidium bromide and using fluorescence microscopy [26] in MCF-7 cell line as sensitive cells to the most potent compounds **1c** and **1d**. MCF-7 cells grown in 12-well plates (50,000 cells/well) were treated with and without IC<sub>50</sub> concentration of compounds **1c** and **1d** for 24 h. After treatment, cells were harvested and washed three times with phosphate buffer saline (PBS). Then, the cells were stained with 100 µL of a mixture of acridine orange and ethidium bromide (1:1, 100 µg/mL) solutions. Stained cell suspension (10 µL) was placed on a clean microscope slide and covered with a coverslip. The cells were immediately analyzed by Axoscope 2 plus fluorescence microscope from Zeiss (Germany). Acridine orange stains live cells green while ethidium bromide intercalate in DNA of cells with an altered cell membrane and emits red fluorescence. Ethidium bromide staining is due to loss of membrane integrity so the population of late stage of apoptotic cells was recognized.

### Flow cytometry analyses of the apoptotic cells with annexin V-PE and 7-AAD (7-aminoactinomycin D) double staining

For detection of early stage of apoptosis and percentage of apoptotic cells in MCF-7 cell line treated with **1c** and **1d**, annexin-V-PE/7-AAD double staining kit (BD Pharmingen™, USA) was used [27]. Staining with annexin V-PE is typically used in conjunction with a vital dye such as 7-AAD to identify early apoptotic cell (7-AAD negative, PE annexin V positive). Viable cells with intact membranes in early apoptosis phase exclude 7-AAD whereas the membranes of dead and damaged cells (late apoptosis and necrosis) are permeable to 7-AAD. Briefly, 24 h

following treating the MCF-7 cells with and without compounds **1c**, **1d**, and etoposide at the concentration of their IC<sub>50</sub>, cells were collected and washed twice with cold PBS and resuspended in the binding buffer (100 µL of calcium buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl<sub>2</sub>). Then, the cells were double stained with 5 µL of annexin V-PE and 5 µL of 7-AAD solution. Finally, the samples were incubated for 15 min at room temperature and then analyzed by flow cytometry.

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